UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE FARMACIA

Departamento de Farmacia y Tecnología Farmacéutica

Hidrogeles acrílicos con ciclodextrinas copolimerizadas y ciclodextrinas colgantes como sistemas de liberación de medicamentos

José Fernando Rosa dos Santos

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Llegado el momento de dar los retoques finales al trabajo de estos últimos años, quiero expresar mi más sincero y profundo agradecimiento a mis directores de Tesis, los profesores Carmen Alvarez Lorenzo y Juan José Torres Labandeira, quienes con su apoyo, dedicación y paciencia durante estos años han hecho que llegara a buen puerto.

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CARMEN ISABEL ALVAREZ LORENZO Y JUAN JOSÉ TORRES LABANDEIRA, PROFESORES TITULARES DE FARMACIA Y TECNOLOGÍA FARMACÉUTICA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA,

CERTIFICAMOS: Que la presente memoria titulada "Hidrogeles acrílicos con ciclodextrinas copolimerizadas y ciclodextrinas colgantes como sistemas de liberación de medicamentos" elaborada por el Licenciado en Farmacia Don José Fernando Rosa dos Santos ha sido realizada bajo nuestra dirección, en el Departamento de Farmacia y Tecnología Farmacéutica y, hallándose concluida, autorizamos su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y, para que conste, expedimos y firmamos la presente certificación en Santiago de Compostela a 7 de octubre de 2009.

C. I. Alvarez Lorenzo J. J. Torres Labandeira

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1. Introducción

1. INTRODUCCIÓN

Para que un fármaco se absorba y pueda alcanzar su lugar de acción debe presentar un balance adecuado entre su solubilidad en medio acuoso y su capacidad para atravesar las membranas biológicas. La mayoría de los fármacos considerados como esenciales por la Organización Mundial de la Salud, y de las nuevas entidades químicas candidatas a fármacos, presentan un carácter marcadamente lipofílico y, consecuentemente, una baja hidrosolubilidad (*Lipinski, 2000; Takagi y col., 2006*). También son numerosos los fármacos que plantean problemas de estabilidad y de toxicidad. Todo ello dificulta el desarrollo de sistemas farmacéuticos eficaces y seguros, y obliga a buscar nuevas estrategias que permitan alcanzar las concentraciones requeridas en el lugar de acción sin comprometer la seguridad del tratamiento. En este contexto, el diseño de sistemas de liberación de fármacos adaptados a las propiedades fisicoquímicas y farmacológicas de los fármacos y capaces de regular su liberación en respuesta a la evolución de ciertos procesos patológicos despierta un interés creciente *(Lee y col., 2007; Alvarez-Lorenzo y Concheiro, 2008)*. Las ciclodextrinas (CDs) resultan particularmente atractivas para el desarrollo de dispositivos avanzados por su capacidad para formar complejos de inclusión con moléculas de naturaleza

muy diversa (*Davis y Brewster, 2004*). La posibilidad de incorporar CDs a una gran variedad de estructuras poliméricas abre interesantes perspectivas para el desarrollo de sistemas hidrofílicos capaces de cargar fármacos hidrofóbicos o hidrofílicos y cederlos de forma controlada (*Uekama y col., 1998; Rodríguez-Tenreiro y col., 2006*).

1.1. Estructura y propiedades de las ciclodextrinas

Las CDs son oligosacáridos cíclicos constituidos por anillos de seis a doce unidades de glucosa ligadas por enlaces α(1-4) (Fig. 1.1). Desde el descubrimiento, hace más de 100 años, de las ciclodextrinas naturales, α (6 unidades), β (7 unidades) y γ (8 unidades), se han mejorado notablemente las técnicas de producción y purificación y se han puesto a punto numerosos métodos de preparación de derivados, lo que ha permitido incrementar sus aplicaciones en el campo farmacéutico (*Loftsson y Duchene, 2007*). La β-CD es poco soluble en agua por su tendencia a formar puentes de hidrogeno intramoleculares. La sustitución de algunos de sus grupos hidroxilo por otros grupos hidrofílicos o hidrofóbicos conduce a la formación de derivados más hidrosolubles (*Jicsinszky y col., 1996; Szejtli, 1998*). Aunque se encuentran disponibles en el mercado un gran número de derivados, sólo la hidroxipropil-β-CD, la sulfobutil-β-CD y la metil-β-CD cuentan, al igual que la α-, β- y γ-CDs, con la aprobación de las agencias reguladoras para ser utilizadas como excipientes farmacéuticos. La solubilidad, la capacidad de la CD para formar complejos con otras sustancias y otras propiedades de interés práctico dependen del grado de sustitución (*Szejtli, 1998; Uekama y col., 1998; Blanchard y Proniuk, 1999; Brewster y Loftsson, 2007*). El grado de sustitución (*Blanchard y Proniuk, 1999*) de las CDs de calidad farmacéutica es 4.5-7 para la 2-hidroxipropil-β-CD (0.65-1 grupos hidroxipropilo por unidad de glucosa), 7 para la sulfobutil-β-CD, y 4-12 para la β-CD metilada aleatoriamente (0.57-1.8 grupos metilo por unidad de glucosa).

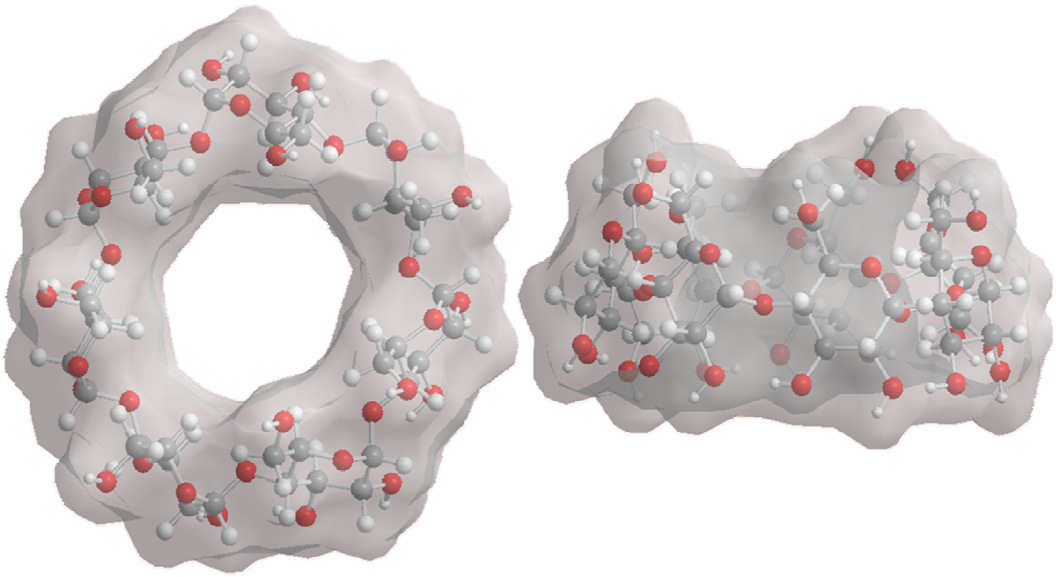

Fig. 1.1. Estructura de la β-ciclodextrina, vistas frontal y lateral.

Las CDs tienen forma de cono truncado con un diámetro máximo comprendido entre 4.7 y 8.3 Å y una altura de 7.9 Å. La cara externa es hidrofílica mientras que la cara interna es hidrofóbica. Las CDs pueden formar complejos de inclusión con moléculas capaces de penetrar total o parcialmente en su cavidad. La complejación se produce como consecuencia de la sustitución de las moléculas de agua que ocupan la cavidad de las CDs, por otras moléculas de polaridad más baja. En la complejación intervienen interacciones hidrofóbicas, electrostáticas y de van der Waals, junto con puentes de hidrógeno y cambios conformacionales, estableciéndose un rápido equilibrio entre las moléculas que se encuentran libres en el medio y las que se alojan en el interior de la cavidad de la CD (*Liu y Guo, 2002*). La adición de cosolventes o polímeros y los cambios de pH o de temperatura pueden desplazar el equilibrio hacia la complejación o la decomplejación (*Thompson, 1997; Loftsson y col., 1999; Loftsson y Másson,*

2001; Perlovich y col., 2003). La formación de complejos con CDs se puede utilizar para corregir las propiedades organolépticas, incrementar la solubilidad aparente y mejorar la estabilidad de los fármacos (*Loftsson y col., 2005a; Szejtli y Szente, 2005; Brewster y Loftsson, 2007*). La naturaleza amorfa de los complejos en estado sólido determina que la velocidad de disolución del fármaco se incremente considerablemente.

Por su elevado tamaño e hidrofilia, las CDs y los complejos CD-fármaco no atraviesan las membranas biológicas (*Irie y Uekama, 1997*). No obstante, la presencia de los complejos en la proximidad de la membrana constituye un reservorio que proporciona una concentración aparente de fármaco muy alta, lo que facilita el paso por difusión del fármaco libre. Además, los complejos difunden mejor que las moléculas de fármaco libre a través de las capas acuosas asociadas a la superficie de las mucosas. A medida que el fármaco va atravesando la membrana, se produce una progresiva decomplejación para que se mantenga el equilibrio de complejación. Además, la complejación puede estabilizar los fármacos evitando su degradación en la zona de absorción. Las CDs pueden actuar también como promotores de la absorción, extrayendo componentes lipófilos de las membranas (*Frijlink y col., 1990*) (Fig. 1.2). Además, los complejos se pueden utilizar para prevenir la incidencia de efectos secundarios locales principalmente, irritación ocular, gastrointestinal o dérmica (*Amdidouche y col., 1994*).

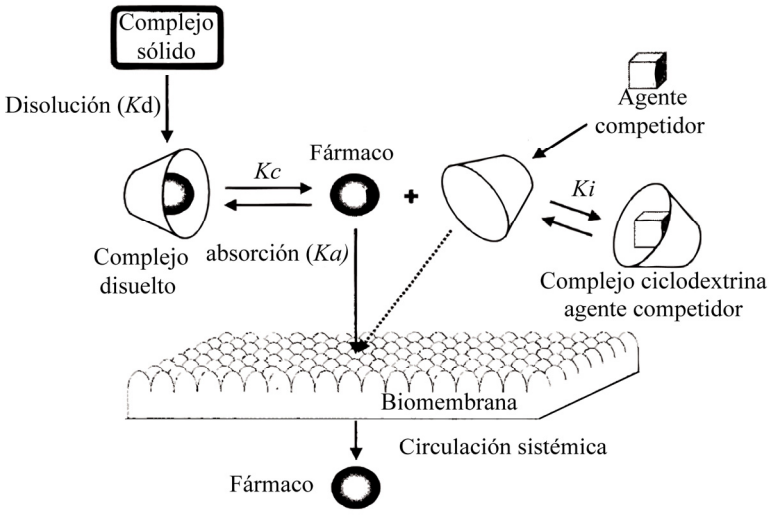

Fig. 1.2. Esquema del proceso de disolución y absorción del fármaco a partir de un complejo de inclusión, y complejación competitiva de los componentes de la membrana (*Uekama, 2004*).

Esta variedad de funcionalidades y aplicaciones junto con la amplia disponibilidad de datos que prueban la seguridad de las CDs (*Irie y Uekama, 1997*) y la progresiva reducción de los precios de estos excipientes, explican que el número de medicamentos que las incorporan se haya incrementado en los últimos años de una manera muy notable (*Stella y Rajewski, 1997; Davis y Brewster, 2004; Szejtli, 2004; Uekama, 2004; Loftsson y col., 2005b; Brewster y Loftsson, 2007*). En la actualidad, están comercializados en el mundo alrededor de cuarenta medicamentos basados en complejos con CDs, para administración oral, sublingual, nasal, ocular, tópica o parenteral, y es previsible que este número se siga incrementando en los próximos años (*Challa y col., 2005; Brewster y Loftsson, 2007; Loftsson y Duchene, 2007*). El interés por las CDs se ha visto

potenciado desde la introducción del Sistema de Clasificación Biofarmacéutica (SCB) de fármacos que se administran por vía oral (*Loftsson y col., 2004*). De acuerdo con el SCB, los fármacos se distribuyen en cuatro clases, según la solubilidad y la velocidad de disolución de la dosis terapéutica y la capacidad para atravesar las membranas biológicas (*Amidon y col., 1995*). El sistema, adoptado por la FDA y por la EMEA, identifica como de Clase I los fármacos que presentan las características de solubilidad y permeabilidad óptimas para ser absorbidos por vía oral (*Gupta y col., 2006*). Las CDs ofrecen la posibilidad de reubicar en la Clase I fármacos de Clase II (baja solubilidad y alta permeabilidad) y fármacos de Clase IV (baja solubilidad y baja permeabilidad) incrementado la solubilidad y la permeabilidad (*Loftsson y col., 2004a*).

Independientemente de la magnitud de la constante de estabilidad del complejo, el fármaco se libera instantáneamente al producirse la dilución en los fluidos biológicos (*Cabral Marques, 1994; Uekama y col., 1994; Stella y col., 1999*). Este fenómeno resulta útil para desarrollar sistemas de liberación inmediata, tanto líquidos como sólidos. La formulación de glucósidos cardiotónicos, analgésicos o antiepilépticos con CDs hidrofílicas conduce a sistemas que se humectan y se disuelven rápidamente, asegurando una absorción inmediata como la que se require en situaciones de emergencia (*Jarvinen y col., 1995*). Por su parte, las CDs con sustituyentes anfifílicos dan lugar a agregados supramoleculares o nanosferas que pueden incorporar grandes cantidades de fármacos hidrofóbicos (complejándolos o interaccionando con complejos previamente formados). Estas nanoestructuras también facilitan que el fármaco se libere rápidamente hacia el medio acuoso (*Géze y col., 2002; Magnusdottir y col., 2002; Loftsson y col., 2004b*). Por otro lado, las CDs se pueden modificar con grupos ionizables o hidrofóbicos para dotarlas de capacidad para retrasar o para prolongar la liberación (*Uekama y col., 1998*). Por ejemplo, las CDs modificadas

con grupos ácido carboxílico, como la 6-O-(carboximetil)-O-etil-β-CD, presentan una solubilidad dependiente del pH y resultan útiles para preparar formas entéricas (*Uekama y col., 1993*). Los conjugados CD-fármaco tienen también un gran potencial en el desarrollo de formas de liberación colónica (*Hirayama y Uekama, 1999*). Para conseguir una liberación lenta con fármacos de elevada hidrosolubilidad se puede acudir a CDs con sustituyentes alquílicos o acilados, si bien su capacidad para controlar la liberación se ve limitada por la decomplejación relativamente rápida que se produce como consecuencia de la dilución de las formulaciones en el medio biológico (*Horikawa y col., 1995; Ikeda y col., 2000*). Puesto que el equilibrio depende fundamentalmente de la concentración local de CD, una estrategia muy atractiva para controlar la liberación consiste en incorporar el complejo a estructuras que minimicen la dilución.

A diferencia de lo que ocurre en los sistemas en los que los complejos CD-fármaco no tienen restringida su movilidad y liberan el fármaco a una velocidad dependiente del proceso de dilución, la incorporación de CDs a entramados poliméricos o hidrogeles permite controlar la liberación regulando la difusión y/o la afinidad del fármaco por las CDs inmovilizadas (*Davis y Brewster, 2005*). Los hidrogeles son materiales muy versátiles que encierran un gran potencial como componentes de sistemas de liberación de medicamentos. Su elevada biocompatibilidad hace que resulten adecuados para la práctica totalidad de las vías de administración (*Peppas y col., 2000*). Los hidrogeles físicamente reticulados controlan la liberación por el efecto que ejerce la viscosidad sobre la difusión. Sin embargo, su capacidad de regulación no es tan elevada como cabría esperar de los altos valores de macroviscosidad (viscosidad aparente) que presentan, puesto que la variable crítica es la viscosidad del microentorno a través del que debe difundir el fármaco (*Alvarez-Lorenzo y col., 1999; Barreiro-Iglesias*

y col., 2001). Como consecuencia de ello, es frecuente que no se consiga un control eficaz de la liberación con hidrogeles muy viscosos. Los hidrogeles químicamente reticulados ofrecen mayores posibilidades de control de la cesión. El tamaño de malla del entramado se puede mantener inalterado en el transcurso del proceso o bien modificarse por efecto de estímulos o por reacciones de degradación enzimática (*Hoare y Kohane, 2007*). Desde un punto de vista práctico, la utilidad de los hidrogeles se ve limitada por la escasa afinidad por los fármacos lipofílicos, que impide su incorporación en cantidad suficiente, y por el deficiente control de la liberación de fármacos hidrofílicos. La incorporación de CDs permite paliar estas limitaciones.

En los apartados siguientes se discuten las estrategias a las que se está acudiendo para desarrollar sistemas de liberación de fármacos: i) dispersión de CDs en hidrogeles físicamente reticulados y ii) unión covalente de CDs a hidrogeles químicamente reticulados. La segunda aproximación implica una mayor restricción en la movilidad de las CDs. En la figura 1.3 se esquematizan algunas modalidades de incorporación de CDs a entramados poliméricos.

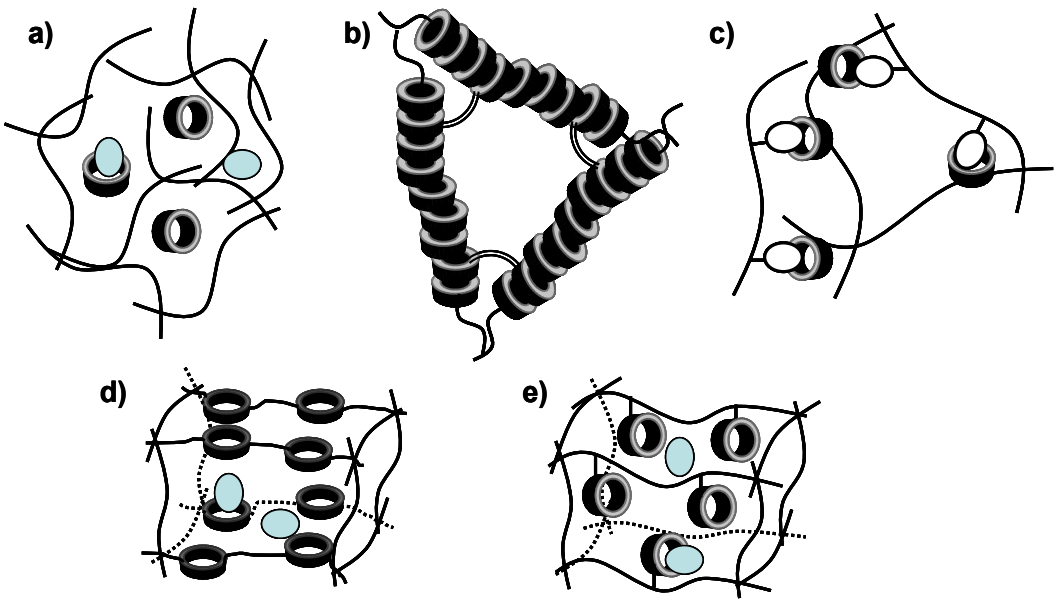

Fig. 1.3. Distintas formas en las que se pueden encontrar las CDs formando parte de entramados poliméricos: a) CDs libres; b) poli(pseudo)rotaxanos con las CDs químicamente unidas; c) CDs formando parte de las cadenas poliméricas y actúando como agentes reticulantes que unen varias cadenas; d) CDs formando parte del entramado tridimensional del hidrogel; y e) CDs "colgantes" de la estructura del entramado.

1.2. CDs dispersas en hidrogeles

La dispersión de CDs en matrices sólidas o semisólidas (Fig. 1.3a) es un recurso muy útil para modificar la velocidad de cesión y modular la biodisponibilidad de los fármacos. Si el fármaco se incorpora en cantidades muy altas, las CDs pueden promover la cesión, incrementando la proporción de fármaco disuelto y en condiciones de difundir. Por el contrario, si el fármaco se encuentra en proporciones bajas, de manera que una vez que el sistema se hidrata la concentración es inferior a su coeficiente de solubilidad, la complejación reduce

la concentración de fármaco libre y dificulta la difusión como consecuencia del elevado tamaño de los complejos (*Bibby y col., 2000*). Por ejemplo, la incorporación de un 1% de 2-hidroxipropil-β-CD (HP-β-CD) o β-CD aleatoriamente metilada (M-β-CD) a hidrogeles físicos de hidroxipropilmetilcelulosa (HPMC 4000 cPs) facilita la solubilization de la melatonina y promueve su absorción a través de la mucosa nasal. Si la CD se incorpora en proporciones más elevadas (5-10%), la formación de complejos estables, que difunden con dificultad por su gran tamaño, hace que la absorción nasal disminuya (*Babu y col., 2008*). La incidencia de otros mecanismos, como el impedimento estérico o la interacción de la CD o el fármaco con otros componentes de la formulación, explican los resultados aparentemente contradictorios que se encuentran recogidos en la bibliografía (*Quaglia y col., 2001a; Rao y col., 2001*).

La formación de complejos con CDs permite formular fármacos hidrofóbicos en sistemas poliméricos hidrofílicos (*Werner y col., 2004*). Como regla general, si el fármaco forma un complejo estable con la CD, la velocidad de difusión dentro del entramado es menor que la que presenta el fármaco libre debido a la diferencia de tamaño (*Orienti y col., 1991*). Se han desarrollado modelos matemáticos para interpretar los perfiles de liberación de fármacos desde entramados poliméricos hidratados y estimar la difusividad del fármaco libre y del complejo CD-fármaco. Uno de estos modelos se ha aplicado al sistema constituido por β-CD, polietilenglicol y nicardipino, para el que se ha observado una progresiva reducción de la velocidad de cesión del fármaco a medida que se va incrementado la proporción de CD (*Quaqlia y col., 2001b*). La complejación del fármaco da lugar a estructuras de elevado volumen hidrodinámico, de manera que su difusividad se reduce de $5 \cdot 10^{-8}$ a $1.2 \cdot 10^{-8} cm^2/s$ (*Quaqlia y col., 2001b*). Hay dos situaciones en las que esta tendencia no se manifiesta:

a) cuando la formación del complejo incrementa significativamente la solubilidad del fármaco y facilita su disolución y su salida del entramado polimérico (*Koester y col., 2003*). En este caso, un elevado gradiente de concentración de fármaco permite mejorar significativamente la absorción oral y transdérmica (*Doliwa y col., 2001*). Especialmente ilustrativos son los resultados obtenidos con HP-β-CD y γ-CD y con ibuprofeno, ketoprofeno y prednisolona en hidrogeles de polivinilpirrolidona (PVP) reticulada con ácido polietilenglicoldimetacrílico (*Woldum y col., 2008*). La HP-β-CD y la γ-CD difieren en tamaño, solubilidad y afinidad por estos fármacos. La incorporación de los fármacos se llevó a cabo sumergiendo hidrogeles secos en una disolución saturada de fármaco (hidrogeles control) o en una disolución de complejo CD-fármaco. La relación molar CD:fármaco en la disolución de carga se situó entre 6 y 50, solubilizándose la totalidad de la dosis al encontrarse la CD en exceso. Con la HP-β-CD se consiguió incrementar, con respecto al hidrogel control, 6, 9 y 3 veces la carga de ibuprofeno, ketoprofeno y prednisolona. Con la γ-CD se duplicó la cantidad de prednisolona cargada, pero no se mejoró la carga de ibuprofeno o ketoprofeno debido a la baja solubilidad que presentan los correspondientes complejos. La HP-β-CD comunicó al sistema una mayor rapidez de cesión de los tres fármacos, mientras que la γ-CD no solo no alteró la liberación de ibuprofeno, debido a su baja tendencia a formar complejos, sino que retrasó la cesión del ketoprofeno y la prednisolona. Este último efecto se explica por el gran tamaño y el bajo coeficiente de difusión de los complejos que forma la γ-CD. Cuando la constante de afinidad CD-fármaco es suficientemente alta, no se requiere la preparación previa del complejo para que se produzcan los efectos comentados, al producirse la complejación de forma espontánea dentro del entramado del hidrogel hidratado (*Rao y col., 2001*).

b) cuando el fármaco interacciona fuertemente con el entramado polimérico. Esto es lo que ocurre al formular hidrocloruro de propanolol en geles

de ácido poliacrílico (Carbopol®). El fármaco, de naturaleza catiónica, forma complejos insolubles con las cadenas poliméricas y reduce de forma significativa el grado de hinchamiento y la bioadhesividad de los microgeles. La complejación con la β-CD minimiza las interacciones fármaco-polímero, devolviendo a los microgeles su comportamiento característico e incrementando la velocidad de cesión (*Blanco-Fuente y col., 2002*).

Cuando el fármaco y la CD no forman complejos, las CDs hidrosolubles pueden aumentar la velocidad de cesión ya que, a medida que se disuelven y abandonan la matriz del hidrogel, forman canales por los que puede salir el fármaco. Las CD menos hidrofílicas aumentan la tortuosidad, dificultando la difusión y retrasando la liberación del fármaco (*Bibby y col., 2000*). Una buena prueba del complicado efecto de las CDs en la cesión de fármaco a partir de entramados reticulados físicamente, son los resultados obtenidos al incorporar β-CD e HP-β-CD a geles y comprimidos matriciales de hidroxipropilmetilcelulosa (HPMC K4M) con diclofenaco sódico (soluble en agua) o sulfametizol (poco soluble en agua). Ambos fármacos dan lugar a complejos con constantes de estabilidad para diclofenaco sódico de 100.6 y 115.2 M^{-1} y para sulfametizol 651.8 y 563.9 M^{-1}, con β-CD y HP-β-CD respectivamente (*Pose-Vilarnovo y col., 1999; Pose-Vilarnovo y col., 2001*). La influencia de la β-CD y la HP-β-CD sobre la difusión del fármaco se puso claramente de relieve en los geles preparados con HPMC al 2%, una proporción superior a la concentración de gelificación. En estos sistemas, una relación molar CD:fármaco 0.5:1 aumentó la velocidad de difusión al minimizar las interacciones hidrofóbicas entre el polímero y el fármaco. En cambio, un exceso de CD, en especial de la voluminosa HP-β-CD, dificultó la difusión de los complejos a través de la malla relativamente estrecha del entramado. En los comprimidos matriciales, las CDs actuan también como promotores de la disolución. Para analizar el efecto sobre la cesión del aumento de

la velocidad de disolución y de la reducción de la velocidad de difusión, se prepararon comprimidos matriciales por compresión directa de 100 mg de fármaco y una mezcla de 400 mg formada por HPMC/CD/lactosa en proporciones establecidas siguiendo un diseño "simplex centroide". Una proporción elevada de CD/lactosa aumentó drásticamente la velocidad de cesión de sulfametizol y redujo la velocidad de cesión de diclofenaco sódico (Fig. 1.4), lo que prueba que el predominio de uno u otro efecto depende la hidrofilia del fármaco (*Pose-Vilarnovo y col., 2004*).

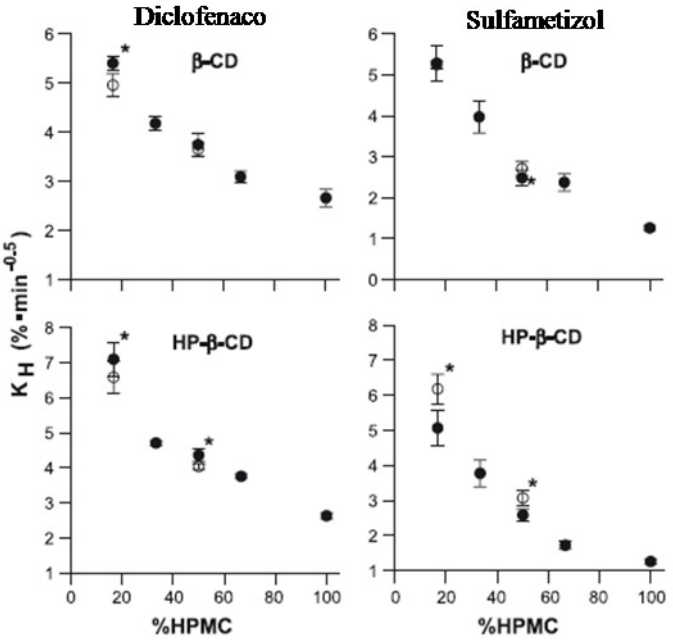

Fig. 1.4. Influencia de la concentración de HPMC sobre la velocidad de cesión de un fármaco a partir de matrices que incorporan distintas proporciones de CD y lactosa (la concentración de polímero se sitúa entre el 16.66% y el 50%). Las formulaciones con mayor proporción de CD/lactosa (simbolos abiertos) presentaron una menor velocidad de cesión de diclofenaco y una mayor velocidad de cesión de sulfametizol (Pose-Vilarnovo y col., 2004).

Otro aspecto a tener en cuenta es la posibilidad de que se produzca una complejación parcial de los componentes poliméricos con la CD, en especial cuando se utilizan copolímeros anfifílicos o polímeros con grupos hidrofóbicos (*Tonelli, 2008*). Por ejemplo, las CDs se pueden utilizar para modular la viscosidad y la respuesta a la luz de polímeros funcionalizados con grupos azobenceno. El azobenceno experimenta cambios de conformación trans/cis cuando se irradia con luz UV. En la oscuridad, los isómeros trans, de naturaleza hidrofóbica, se asocian y actúan como puntos de unión a lo largo de la cadena polimérica, con lo que aumenta la viscosidad del gel. Bajo irradiación, la transformación de trans a cis favorece la rotura de los puntos de unión, ya que la forma cis tiene un carácter más hidrofílico. El isómero trans del azobenceno puede formar complejos con α-CD, inhibiendo su autoasociación e incluso revertiendo el efecto de la radiación UV sobre la viscosidad del sistema (*Zheng y col., 2004*). Por otro lado, el isómero cis puede formar complejos con HP-β-CD. Si los sustituyentes azobenceno forman parte de un copolímero anfifílico, como el poli(N,N-dimetilacrilamida-co-metacriloiloxiazobenceno) (DMA-MOAB), y se añade HP-β-CD se puede alterar considerablemente, en función del grado de irradiación, su capacidad para interaccionar con otros copolímeros anfifílicos y dar lugar a micelas. Este fenómeno puede ser útil para modular la difusión de solutos hidrofílicos a través de hidrogeles (*Alvarez-Lorenzo y col., 2007*).

Las CDs también pueden formar complejos con copolímeros bloque de estequiometría muy superior a 1:1, dando lugar a estructuras en forma de collar denominadas polipseudorotaxanos (*Loethen y col., 2007*). Las CDs se ensartan en las cadenas de polímero acumulándose en las regiones más favorables para la complejación (por ejemplo, la α-CD en los bloques polióxido de etileno y la β-CD en los bloques polióxido de propileno) (*Okada y col., 1999; Kidowaki y col., 2006; Kikuzawa y col., 2008*). Si los extremos del polímero se bloquean con

grupos voluminosos de manera que las CDs no puedan abandonar el polímero, se obtienen polirotaxanos. Las interacciones intermoleculares entre unidades de CD en los polirotaxanos pueden dar lugar a la formación de superestructuras en forma de nanotubos. También se ha propuesto la reticulación de CDs de polirotaxanos adyacentes (*Karaky y col., 2008*) (fig. 1.3b) o la unión química de CDs al final de cadenas poliméricas (*Tamura y col., 2007*) para obtener geles con puntos de reticulación deslizantes. Un adecuado diseño de los polirotaxanos permite una amplia modulación de las propiedades del hidrogel, lo que abre interesantes posibilidades en el ámbito de la biomedicina (*Huang y Gibson, 2005; Loethen y col., 2007; Yamashita y col., 2008*). Aunque el número de estudios sobre la incidencia de los polirotaxanos sobre la solubilidad y difusividad de fármacos es todavía reducido, los resultados obtenidos hasta el momento indican que la complejación espontánea de copolímeros bloque con CDs aumenta la concentración crítica micelar del copolímero y reduce el número de micelas y de cavidades de CD libres para albergar moléculas de fármaco (*Ooya y Yui, 1999; Rodriguez-Pérez y col., 2006; Rodriguez-Pérez y col., 2007*). Esto da lugar a una menor eficacia de solubilización de fármacos hidrofóbicos. Además, en el caso de los copolímeros bloques de óxido de polietileno/óxido de polipropileno sensibles a temperatura, como los PEO-PPO-PEO comercializados bajo la denominación poloxamer o Pluronic®, se producen cambios importantes en la temperatura de transición sol-gel y en la viscoelasticidad de los geles (*Gonzalez-Gaitano y col., 1997; Rodriguez-Pérez y col., 2006; Li y Loh, 2008*). La adición a dispersiones de Pluronic F127 (15% p/v) de HP-β-CD y M-β-CD (5% p/v) da lugar a un incremento de 5 y 15ºC, respectivamente, en la temperatura de gelificación y reduce significativamente el valor de G´ (módulo elástico o almacenamiento) y de G´´ (módulo viscoso o pérdida) de los geles. Además, el Pluronic F127 desplaza fácilmente las moléculas huésped de la cavidad de las CDs, aumentando la proporción de fármaco libre en el medio (*Nogueiras-Nieto y col., 2009*). La

información disponible hasta el momento prueba la necesidad de identificar la naturaleza y la estequiometría de los complejos cuando se preparan sistemas ternarios fármaco-polímero-CD, una práctica habitual en tecnología farmacéutica.

Un paso adelante en este campo es la preparación de geles estables usando un mecanismo "llave-cerradura" o "cremallera", en el que CDs unidas covalentemente a la cadena polimérica reconocen ciertos grupos de otros polímeros, originado un entramado tridimensional (Fig. 1.3c). Esta reticulación mediada por CD aumenta de forma notable la viscosidad del sistema, dando lugar a entramados que exhiben un comportamiento intermedio entre el de un hidrogel reticulado físicamente, en el que las interacciones son reversibles, y el de un hidrogel reticulado químicamente que es estable frente a la dilución. Este fenómeno se ha observado al mezclar: i) un polímero con CDs "colgantes" y un polímero con cadenas laterales hidrofóbicas de 4-tert-butilanilida (*Wenz y col., 2000*); ii) conjugados de quitosano-CD y quitosano con grupos adamantilo o polietilenoglicol (*Auzely-Velty y Rinaudo, 2002*); iii) poli(acrilamida)-CD y poli(acrilamida) con anillos aromáticos (*Hashidzume y col., 2005*); y iv) polímeros de β-CD (con epiclorhidrina) y poli(N-isopropilacrilamida) con grupos adamantilo o dodecilo (*Wintgens y col., 2005*). Un autoensamblaje espontáneo se produce, por ejemplo, entre polímeros de β-CD (poli-β-CD) y dextranos con cadenas alquílicas colgantes (Fig. 1.5). Al mezclar disoluciones acuosas de ambos polímeros en concentraciones de 6.6–7.5% p/p se produce una rápida separación de fases. La fase gel presenta una elevada concentración de ambos polímeros, con valores de G′ y G′′ de 400-500 Pa y 1200-1400 Pa, respectivamente (*Daoud-Mahammed y col., 2007; Wintges y col., 2008*). Concentraciones más bajas de los polímeros (0.1-1% p/p) dan lugar a la formación de partículas nanométricas estables (*Gref y col., 2006*). Si los polímeros cuentan con grupos ionizables, se pueden conseguir geles de viscosidad variable en función del pH del medio (*Gosselet y col., 2005*). Los hidrogeles se pueden cargar con moléculas que

forman complejos con poli-β-CD antes de mezclar con la disolución de dextranos. Las cavidades vacías, es decir las que no contienen fármaco, estarán disponibles para albergar las cadenas alquílicas y actuar como puntos de unión entre ellas. Estos geles proporcionan perfiles de liberación sostenida de benzofenona y tamoxifeno durante más una semana. Además, la dinámica de complejación polímero-CD, permite administrar el gel mediante inyección a través de agujas relativamente finas (0.838 mm de diámetro interno). Una ligera presión del sistema en la jeringa da lugar a la decomplejación y al descenso de la viscosidad, fluyendo fácilmente. Al abandonar la aguja, los geles recuperan los valores iniciales de G´ y G´´ en pocos segundos (*Daoud-Mahammed y col., 2007*). Estas características junto con una excelente biocompatibilidad aseguran un futuro muy prometedor en el campo biomédico para estos sistemas de gelificación *in situ*.

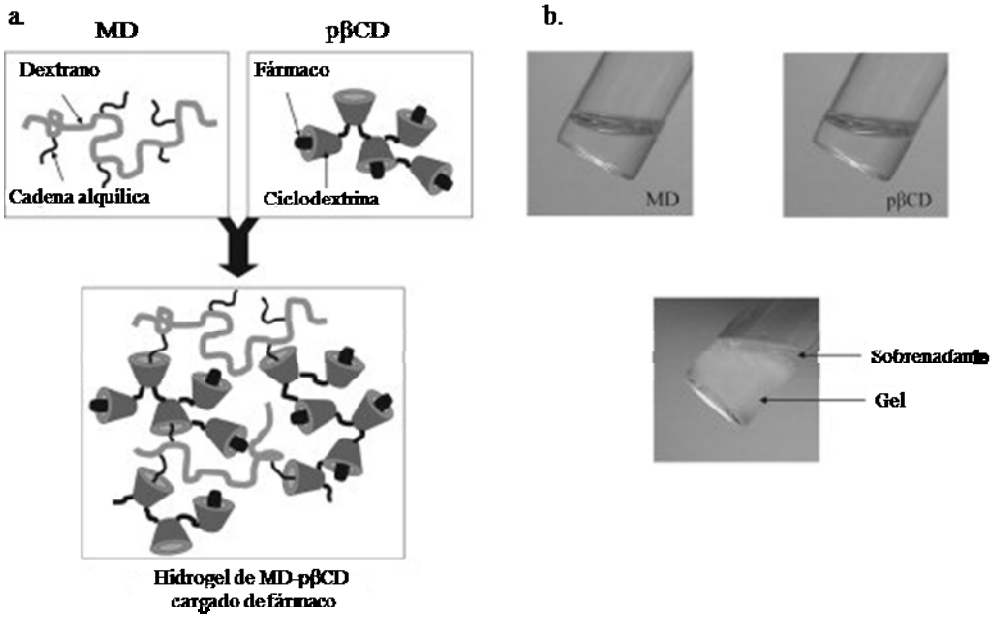

Fig. 1.5. (a) Interacción espontánea de un dextrano alquil-modificado (MD) con poli-β-CD para dar lugar a un gel. Algunas cadenas alquílicas del MD forman complejos con las CDs del poli-β-CD. Las CDs libres pueden formar complejos de inclusión con fármacos hidrofóbicos. (b) Aspecto de las disolución de MD, poli-β-CD y de la mezcla de ambas transcurridos 5 segundos (Daoud-Mahammed y col., 2007).

1.3. Hidrogeles con CDs integradas en su estructura

Se pueden preparar hidrogeles con las CDs formando parte de la estructura de un entramado químicamente reticulado, acudiendo a alguno de los procedimientos siguientes: a) reticulación directa de las CDs (condensación con un agente reticulante), b) copolimerización de las CDs con comonómeros acrílicos o vinílicos, y c) preparación de un entramado polimérico y posterior anclaje de las CDs. La característica principal de los entramados de CDs es que, cuando entran en contacto con un medio acuoso, las CDs no se diluyen, a diferencia de lo que ocurre con las disoluciones y con los hidrogeles reticulados físicamente. El volumen de agua que puede entrar en un hidrogel reticulado químicamente está limitado por el propio entramado y, dado que las CDs se encuentran covalentemente unidas, el hidrogel hincha sin disolverse y sin perder componentes. Esto genera un microambiente rico en cavidades de CD disponibles para interaccionar con moléculas de fármaco. En este tipo de entramados, la afinidad CD-fármaco se convierte en el principal factor responsable del control de la liberación. Cuando una molécula de fármaco se decompleja de una cavidad de CD, encuentra en su entorno otras cavidades disponibles para formar un nuevo complejo. Un hidrogel de CD se puede ver como un entramado constituído por muchas cavidades, a partir del cual se libera el fármaco decomplejándose y complejándose sucesivamente con las CDs que encuentra en su camino hacia la superficie del hidrogel (Fig. 1.6). El desplazamiento del fármaco será más o menos rápido dependiendo del grado de ocupación de las cavidades del entramado. Cuantas más cavidades estén ocupadas, menos etapas de complejación experimentará el fármaco hasta llegar a la superficie, de manera que la velocidad de cesión será mayor. A medida que avanza el proceso de cesión, el número de cavidades disponibles para formar complejos con el fármaco se va incrementando. Ello hace posible que algunas moléculas que se habían liberado previamente puedan ser recaptadas desde el medio por el hidrogel. En consecuencia, los

hidrogeles de CD poseen características únicas para retener fármacos y pueden resultar muy útiles en el desarrollo de sistemas de liberación controlada. Es importante destacar que las CD covalentemente unidas al entramado no ven reducida su capacidad para formar complejos, sino que incluso se puede incrementar, especialmente si la molécula a complejar es grande y se requieren varias CDs para satisfacer la estequiometría del complejo (*Szeman y col., 1987; Crini y col., 1988a; Layre y col., 2002; Li y col., 2004; Liu y col., 2004a; Gazpio y col., 2008; Qian y col., 2008*). Las estrategias a seguir para el desarrollo de entramados de CDs reticulada se describen en los apartados siguientes.

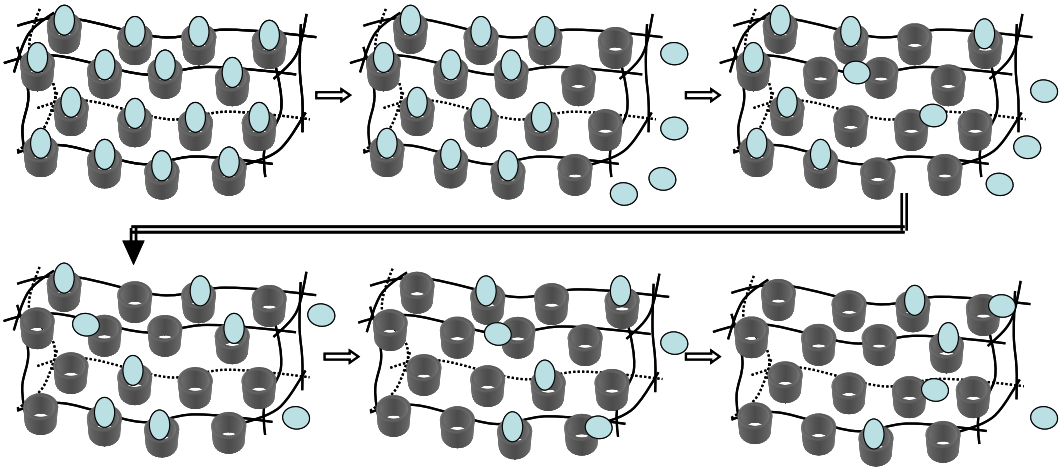

Fig. 1.6. Liberación de un fármaco a partir de un entramado de CDs químicamente reticuladas. El fármaco abandona una cavidad para ocupar la siguiente hasta llegar a la superficie del entramado.

a) Reticulación directa de las CDs

Los primeros intentos de obtener polímeros e hidrogeles de CDs se basaron en reacciones de condensación de los grupos hidroxilo de CDs naturales o de los grupos amino o ácido carboxílico de CDs funcionalizadas, utilizando

agentes reticulantes di- o multifuncionales tipo aldehído, cetona, isocianato o epóxido (epichlorhidrina) (*Crini y Morcellet, 2002*). Aunque la condensación transcurre de manera espontánea, normalmente se incorpora un catalizador (generalmente una base, o un ácido en el caso del glioxal o del glutaraldehído; *Xu y col., 2003*) para incrementar la velocidad del proceso. El agente reticulante más utilizado es la epiclorhidrina (EPI). En medio alcalino, los dos grupos funcionales de la EPI pueden reaccionar entre ellos o con los grupos hidroxilo de las CDs. Esto da origen a una mezcla de CDs reticuladas unidas por cadenas cortas de EPI polimerizada (*Kobayashi y col., 1989; Crini y col., 1998b*) (Fig. 1.7). Los hidrogeles de EPI-CD (generalmente microgeles) pueden hinchar de forma considerable en medio acuoso. Controlando la reacción (por ejemplo, parando la reticulación en una determinada etapa) es posible obtener polímeros de CD hidrosolubles (*Li y col., 2004*). La relación EPI:β-CD determina la proporción de cavidades de CD disponibles para interaccionar con el fármaco; alcanzándose el máximo con hidrogeles que contienen un 50% de β-CD (*Velaz y col., 2007*).

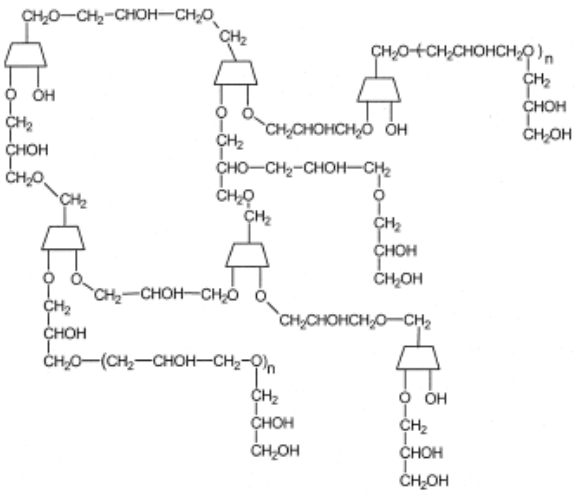

Fig. 1.7. Hidrogel de EPI-CD (Kobayashi y col., 1989).

Las aplicaciones de los microgeles de EPI-CD como adsorbentes para extraer fármacos y moléculas hidrofóbicas del agua (*Orprecio y Evans, 2003; Crini, 2005; Crini, 2008*), para separar compuestos en matrices complejas (*Su y Yang, 1991; Schneiderman y col., 2000; Scriba, 2008; Astray y col., 2009*) o en análisis cromatográfico (*Wiedenhof y col., 1969; Hoffman, 1973*) y para separar enantiómeros a partir de mezclas racémicas (*Harada y col., 1978; Thuaud y col., 2002; Wang y col., 2007*) han sido objeto de numerosos estudios. Para fines biomédicos, se requiere en general una mayor hidrofília, una estructura más flexible y una propiedades mecánicas más versátiles. Para dotar de estas características a los entramados EPI-CD se acude a la combinación con otros polímeros y/o agentes reticulantes, como por ejemplo el alcohol polivinílico (PVA) y el etilenglicolbis(epoxipropil)éter (*Szejtli y col., 1978*). La incorporación de grupos carboximetilo o acetilo permite regular la hidrofilia sin comprometer la capacidad de las CDs para formar complejos de inclusión, como se comprobó con fármacos desinfectantes como el lactato de etacridina, el verde brillante, el ácido fucsínico o el cloruro de cetilpiridino (*Fenyvesi y col., 1996*).

Con el fin de dotar a los conjugados EPI-CD de sensibilidad a la temperatura, se ha propuesto la incorporación de poli(N-isopropilacrilamida) (PNIPA) aplicando una de las dos estrategias siguientes:

a) unión directa de PNIPA a β-CDs previamente reticuladas para dar origen a hidrogeles que hinchan a temperatura ambiente y se contraen a 37ºC. El uso de marcadores fluorescentes mostró que, por debajo de la temperatura de transición, la complejación con las CDs reticuladas es más favorable (K_a 100 veces mayor) que la que se observa para disoluciones de CD, lo que se puede atribuir al microambiente hidrofóbico que generan las cadenas de PNIPA en torno a la β-CD (Fig. 1.8). Por el contrario, a 37ºC, el colapso de las cadenas de PNIPA origina impedimentos estéricos que limitan el acceso de los marcadores a las cavidades de las CDs (*Nozaki y col., 1997*).

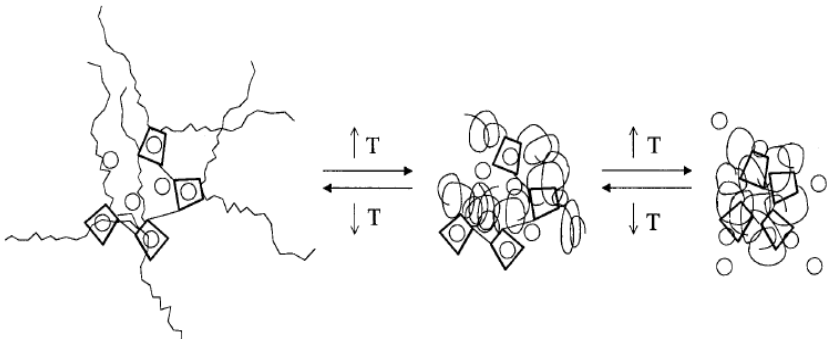

Fig. 1.8. Inclusión de un marcador fluorescente (o) en un hidrogel termosensible de CDs reticuladas y que tienen ancladas cadenas de poli(N-isopropilacrilamida) (Nozaki y col., 1997).

b) preparación de entramados interpenetrados (IPN) o semiinterpenetrados (semi-IPN) de EPI-CD y PNIPA. Por ejemplo, se sintetizó un hidrogel de PNIPA reticulado con N,N´-metilenbis(acrilamida) en presencia de un entramado de EPI-β-CD. El IPN mantuvo la temperatura de transición del PNIPA pero, a diferencia de los hidrogeles convencionales de PNIPA, mostró un buen control de la liberación de ibuprofeno gracias a la capacidad de complejación de las CDs (*Zhang y col., 2005*). Resultados similares se han obtenido con propanolol e hidrogeles semi-IPN de β-CD ancladas a polietilenamina y PNIPA reticulado (*Zhang y col., 2008*).

Por otra parte, se han preparado microgeles sensibles a cambios de pH, interpenetrando microgeles de EPI-CD-PVA con poli(ácido metacrilico) (PMAA) (*Liu y col., 2004b*). A pesar de que los microgeles se colapsan a pH 1.4 e hinchan a pH 7, se observó una velocidad de liberación de naranja de metilo mucho mayor a pH ácido. Este comportamiento se explica porque a pH neutro el naranja de metilo no se encuentra ionizado y su afinidad por la β-CD es superior en un orden

de magnitud. Por lo tanto, la cesión depende del efecto del pH sobre las interacciones fármaco-CD y no sobre el hinchamiento macroscópico del IPN.

El elevado número de grupos hidroxilo de las CDs las hace muy útiles para desarrollar entramados EPI-CD sensibles a campos eléctricos. Estos materiales inteligentes experimentan cambios rápidos y reversibles en sus propiedades reológicas bajo la acción de pequeños campos eléctricos; sin embargo, no resisten un fuerte campo eléctrico durante un tiempo prolongado y la polarización está limitada por la rigidez y la alta densidad de CDs (*Gao y Zhao, 2003*). La incorporación de almidón durante el proceso de reticulación ha permitido obtener entramados que, si se mezclan con aceite de silicona, presentan buenas propiedades electroreológicas (*Gao y Zhao, 2004*).

Algunos hidrogeles de EPI-CD muestran una elevada capacidad de retención de solutos debido, no sólo a la formación de complejos de inclusión, sino también al establecimiento de interacciones específicas a través de los numerosos grupos hidroxilo de las CD. Sacando partido de este mecanismo se han desarrollado hidrogeles selectivos para creatinina (*Tsai y Syu, 2005*). Estos hidrogeles se preparan a pH alcalino en presencia de creatinina. En estas condiciones los OH-6 están ionizados y pueden interaccionar electrostáticamente con los grupos amino de la creatinina. Una vez formado el hidrogel y eliminada la creatinina, se mantiene la conformación de las CDs, lo que permite la recaptación selectiva de creatinina de medios acuosos. La máxima selectividad se manifiesta con proporciones molares β-CD:creatinina 3:2 y β-CD-EPI 1:10. Los entramados de EPI-CD también se pueden funcionalizar con grupos amonio cuaternario para que actuen como trampas de sales biliares (*Baille y col., 2000*).

El potencial de los hidrogeles EPI-CD se ve limitado por la toxicidad relativamente alta de la EPI. Esto ha motivado una intensa búsqueda de agentes reticulantes adecuados para aplicaciones en biomedicina y farmacia. En esta línea se ha generado una abundante bibliografía sobre la utilización de diisocianatos en la elaboración de macro y microhidrogeles de CDs (*Mocanu y col., 2001*). En la figura 1.9 se muestra la estructura de un entramado de β-CDs reticuladas con hexametilendiisocianato en proporción 1:8 (*Yamasaki y col., 2008*). Estos entramados resultan útiles en la eliminación de sustancias toxicas (fenol o colorantes orgánicos) de aguas residuales (*Ozmen y col., 2008*). Los hidrogeles de β-CD y diaminopoli(etilenglicol), reticulados con hexametilendiisocianato son muy hidrofílicos, biocompatibles y capaces de cargar y ceder de forma controlada estradiol, quinina o lisozima (*Salmaso y col., 2007*).

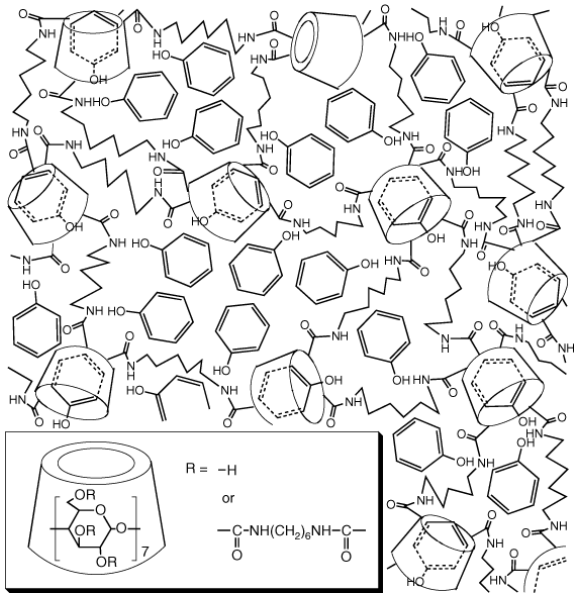

Fig. 1.9. Adsorción de fenol, procedente de aguas residuales, en esferas elaboradas por reticulación de β-CD con hexametileno diisiocianato (Yamasaki y col., 2008).

Los diisocianatos también se han empleado para obtener polímeros hidrofílicos hiperramificados de CDs con capacidad para formar complejos (*Chen y col., 2003*) y partículas nanoporosas de CDs que captan rápidamente solutos del medio acuoso y los ceden a fases orgánicas (*Ma y Li, 1999*). La combinación de esta aproximación con la tecnología de moldeado molecular (*molecular imprinting*) permite mejorar la capacidad de retención y la selectividad de la carga y conseguir un mejor control de la cesión (*Alvarez-Lorenzo y Concheiro, 2004; Alvarez-Lorenzo y Concheiro, 2006*). El grupo de Asanuma y Komiyama ha evaluado ampliamente las posibilidades que ofrece el moldeado molecular, para potenciar la capacidad de los entramados de β-CD reticulados con tolueno-2,4-diisocianato en la separación selectiva de moléculas con actividad biológica y en la extracción de contaminantes de efluentes líquidos (*Asanuma y col., 2004*). La reticulación en presencia de colesterol o estigmasterol conduce a la formación de entramados en los que dímeros o trímeros de β-CD actúan cooperativamente para atrapar cooperativamente moléculas esteroídicas de gran tamaño. Una vez completada la polimerización y retiradas las moléculas que han actuado como moldes, los hidrogeles imprinted (MIP) pueden servir para captar de un medio acuoso colesterol y estigmasterol, mostrando una afinidad mucho menor por otras estructuras químicamente relacionadas (Fig. 1.10) (*Hishiya y col., 1999; Hishiya y col., 2002*).

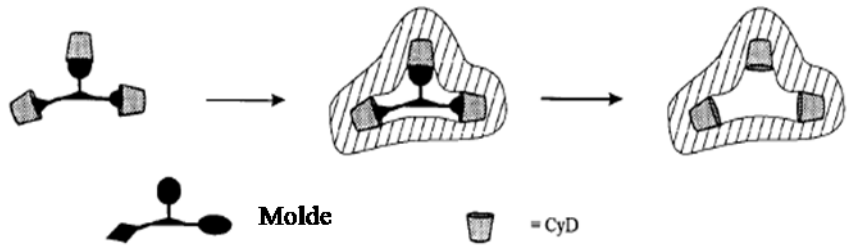

Fig. 1.10. Representación esquematica de un MIP de CD (Hishiya y col., 1999).

La naturaleza del agente reticulante afecta considerablemente a la afinidad de las moléculas huésped por las CDs. Por ejemplo, entramados de β-CD preparados con EPI, cloruro de succinilo, hexametilendiisocianato o tolueno-2,4-diisocianato mostraron mayor capacidad de captación de 1-naftol, que los que se prepararon sustituyendo β-CD por sacarosa, lo que prueba que se forman complejos de inclusión (*Garcia-Zubiri y col., 2007*). La reticulación de β-CD con diisocianatos conduce a la obtención de hidrogeles con menor tamaño de malla y grado de hinchamiento, en comparación con los obtenidos con EPI. En los entramados reticulados con diisocianatos las interacciones hidrofóbicas inespecíficas se producen con más facilidad, mientras que la complejación con CD se ve dificultada. En general los entramados de CDs presentan una mayor capacidad de captación de contaminantes, en particular de fenoles, que los adsorbentes comerciales (*Romo y col., 2008*). Estos hechos, junto con la baja toxicidad, la posibilidad de reciclaje y el bajo coste determina que los entramados de CD tengan un gran potencial para la eliminación de contaminantes del agua (*Crini, 2003; Crini, 2005*).

Para desarrollar microcápsulas recubiertas con β-CD reticuladas se ha utilizado una técnica de emulsión-polimerización con cloruro de diacilo (*Pariot y col., 2000*). Las moléculas huésped acceden rápidamente a las cavidades de las CDs, completandose la carga en 5 minutos, y las microcápsulas sostienen la cesión de propanolol durante varias horas (*Pariot y col., 2002*).

La condensación con ácidos policarboxílicos (Fig. 1.11) constituye una aproximación limpia para obtener de entramados de CD reticulada, aunque tiene el inconveniente de que hay que eliminar el agua que se genera en la esterificación, aplicando vacío o temperaturas superiores a 140ºC (*Martel y col., 2005*). Las CD naturales se pueden poliesterificar con ácido cítrico, ácido 1,2,3,4-

butanotetracarboxílico o poli(ácido acrílico) (PAA), pero no con ácidos dicarboxílicos. Este hecho pone de manifiesto la necesidad de utilizar compuestos que cuenten, al menos, con tres grupos carboxílicos separados por dos o tres átomos de carbono, y fosfato (NaH_2PO_4) que actua como catalizador formando un intermediario anhidro cíclico de poli(ácido carboxílico) capaz de reaccionar con las CDs.

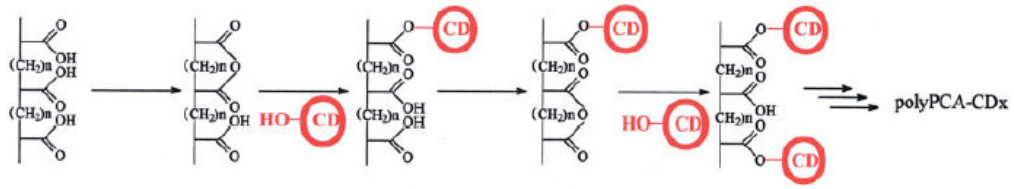

Fig. 1.11. Estructura de un entramado de CDs reticuladas por condensación con poli(ácido carboxílico)s (Martel y col., 2005).

La capacidad de las CDs para reaccionar con grupos epóxido en condiciones suaves (*Komiyama y Hirai, 1987; Zhang y Wang, 2004a*) ha llevado a desarrollar procedimientos para unir CDs en un solo paso. Utilizando etilenglicoldiglicidiléter (EGDE) se pueden obtener hidrogeles viscoelásticos de una forma rápida y predecible (*Alvarez-Lorenzo y col., 2006a; Rodriguez-Tenreiro y col., 2006*). El EGDE es un agente reticulante de baja toxicidad que cuenta en su estructura con dos grupos epóxido de reactividad similar, que pueden reaccionar simultáneamente con los grupos hidroxilo de las CDs o de un polisacárido lineal (*Yui y col., 1992*). La reacción, catalizada por OH^-, requiere una temperatura mínima de 50ºC para que se pueda completarla en unas pocas horas y no se vea comprometida la estabilidad de las CDs (*Rodriguez-Tenreiro y col., 2006*). La mayoría de los grupos glicidileter del EGDE se consumen durante la reacción y, si quedase alguno en el hidrogel, un lavado con una disolución

acuosa de HCl 0.01M es suficiente para que se abran los anillos glicidilo. En consecuencia, los hidrogeles presentan una elevada biocompatibilidad (*Huang y col., 1998*). Para que se formen hidrogeles de HP-β-CD se requiere como mínimo un 10% p/p de HP-β-CD y un 14.28% p/p de EGDE. Estas proporciones permiten que 2/3 de los grupos hidroxilo de cada CD reaccionen con el agente reticulante. Los hidrogeles de CD son transparentes y hinchan en agua hasta 1000% p/p, comportándose como superabsorbentes muy eficaces. También se pueden incorporar, durante el proceso de reticulación, éteres de celulosa (formados por unidades glucopiranosa similares a las de las CDs) con el fin de modular las propiedades mecánicas y de cesión de fármaco. El contenido en HPMC se debe situar entre la concentración de solapamiento (0.2 p/p-%) y la de entrecruzamiento (1 p/p-%) de manera que se produzca una distribución homogénea de los componentes. Los hidrogeles de HP-β-CD y de M-β-CD pueden incorporar cantidades de diclofenaco sódico y de estradiol de 2 a 500 veces superiores a las que se conseguirían si el fármaco se alojase únicamente en la fase acuosa del hidrogel. El papel clave de las CDs en el control de la liberación se puso de manifiesto por la estrecha correlación entre los valores de la constante de complejación fármaco-CD, el coeficiente de reparto del fármaco entre el entramado de CDs y el agua, y la velocidad de cesión (Fig. 1.12).

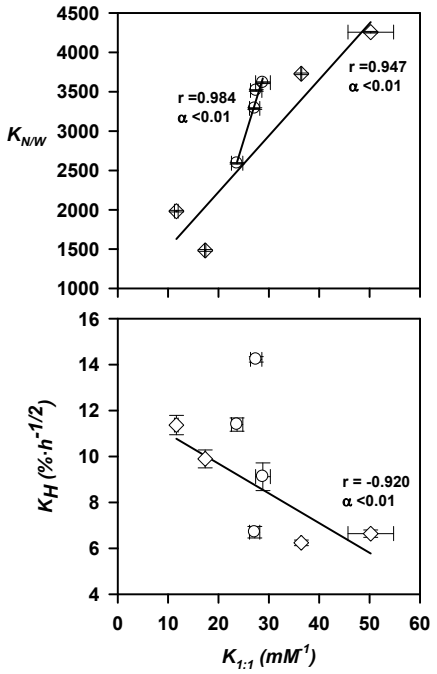

Fig. 1.12. Dependencia del coeficiente de reparto entramado/agua ($K_{N/W}$) y de la constante de liberación (K_H) del estradiol a partir de hidrogeles de HP-β-CD (◊) y M-β-CD (○) respecto de las constantes de estabilidad de los complejos ($K_{1:1}$) (Rodriguez-Tenreiro y col., 2007a).

En el caso del estradiol, se consiguieron perfiles de cesión sostenida durante una semana (*Rodriguez-Tenreiro y col., 2007a*). Aunque la carga y la liberación de fármaco están controladas principalmente por la constante de afinidad fármaco/CD, la incorporación de los éteres de celulosa dota a los hidrogeles de mayor flexibilidad y acelera ligeramente el proceso de cesión (Fig. 1.13).

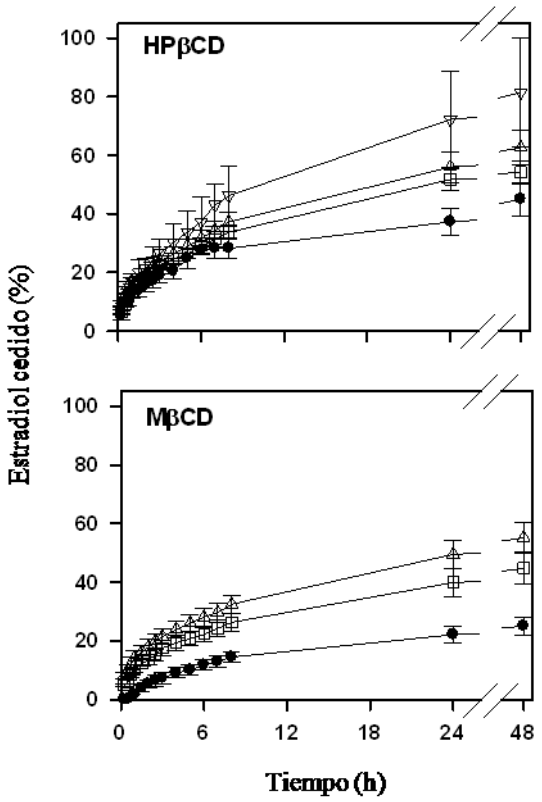

Fig. 1.13. Perfiles de cesión del estradiol a partir de hidrogeles de HP-β-CD o M-β-CD preparados con 20% de CD (●), 20% CD – 0.25% HPMC (□), 25% CD – 0.25% HPMC (Δ), y 30% CD – 0.25% HPMC (▽)(Rodriguez-Tenreiro y col., 2007a).

La versatilidad del procedimiento de reticulación con EGDE se pone de manifiesto por la posibilidad de preparar hidrogeles mixtos de CDs y diversos polímeros relacionados estructuralmente. Se han preparado hidrogeles de HP-β-CD (20%) con 0.4 ó 0.8% de HPMC, metilcelulosa (MC), hidroxipropilcelulosa (HPC), carboximetilcelulosa sódica (CMCNa) y dextrano para cargar sertaconazol (*Lopez-Montero y col., 2009*). Este fármaco es activo frente a *Candida albicans*, pero su reducida solubilidad acuosa dificulta el desarrollo de formulaciones

eficaces. Los hidrogeles de HP-β-CD proporcionan un microambiente rico en cavidades de CD que son las responsables de la carga y control de la cesión del fármaco. Todos los hidrogeles se comportaron como superabsorbentes, si bien los preparados con MC, CMCNa o HPC presentaron menor grado de hinchamiento debido a que estos éteres de celulosa son menos hidrofílicos que la HP-β-CD y, además, dan lugar a un mayor grado de reticulación al reaccionar con EGDE. La dureza y la compresibilidad de los hidrogeles preparados con HPMC o dextrano resultó ser similar a los de los geles preparados únicamente con HP-β-CD (4.2 N·mm y 3.1 N·mm, respectivamente). La incorporación de otros polisacáridos provocó un aumento en estos parámetros (hasta 9.4 N·mm y 8.7 N·mm), lo que confirma una mayor densidad del entramado en los hidrogeles de HP-β-CD/MC, HP-β-CD/CMCNa y HP-β-CD/HPC. Los hidrogeles se cargaron con sertaconazol por inmersión en suspensiones de fármaco. En general, el autoclavado promovió la incorporación del fármaco sin afectar a las propiedades mecánicas del hidrogel (Fig. 1.14). Todos los hidrogeles dieron lugar a una liberación relativamente rápida en las primeras 24 horas, seguida de una fase de cesión más lenta durante los 4 días siguientes. La efectividad antifúngica del sertaconazol liberado a partir de los hidrogeles se confirmó en cultivos de *Candida albicans* en fase exponencial de crecimiento. Así, los hidrogeles de CD-polisacárido reticulados con EGDE presentan un elevado potencial como vehículos para la administración tópica o mucosal de fármacos antifúngicos.

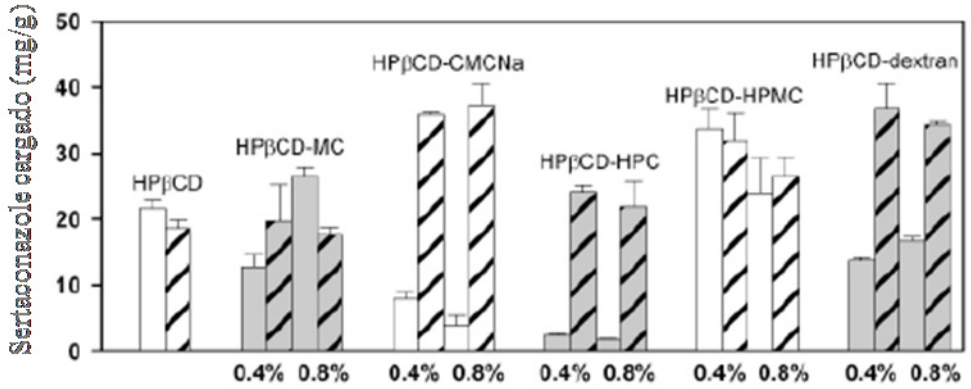

Fig. 1.14. Cantidad de nitrato de sertaconazol incorporada a hidrogeles de HPβCD sintetizados sin polisacáridos o con metilcelulosa (MC), carboximetilcelulosa (CMCNa), hidroxipropilcelulosa (HPC), hidroxipropilmetilcelulosa (HPMC) o dextrano al 0.4 o 0.8%. Las líneas oblicuas indican que los hidrogeles se sometieron a autoclavado durante la carga (Lopez-Montero y col., 2009).

Aplicando un procedimiento similar, se prepararon hidrogeles de HP-β-CD con dominios interpenetrados de poli(ácido acrílico) (PAA, Carbopol®) con el objetivo de combinar la capacidad de respuesta frente a cambios de pH y las propiedades mucoadhesivas del carbopol con la capacidad de formación de complejos de las CDs reticuladas (*Rodriguez-Tenreiro y col., 2007b*). Estos hidrogeles presentan un entramado continuo de CDs y dominios discontinuos de carbopol, resultando en un IPN a microescala (ms-IPN). Los ms-IPNs tienen las siguientes ventajas: i) para su obtención no se requiere la preparación previa de monómeros acrílicos de CD; ii) cuando se hidratan no pierden componentes, como ocurre con los semi-IPNs convencionales; iii) la presencia de PAA reticulado comunica propiedades bioadhesivas y capacidad de respuesta frente a cambios de pH (Fig. 1.15); iv) la estructura discontinua puede facilitar la

movilidad de los entramados lo que dota al conjunto de excelentes propiedades mecánicas; y v) el producto final se obtiene en un solo paso.

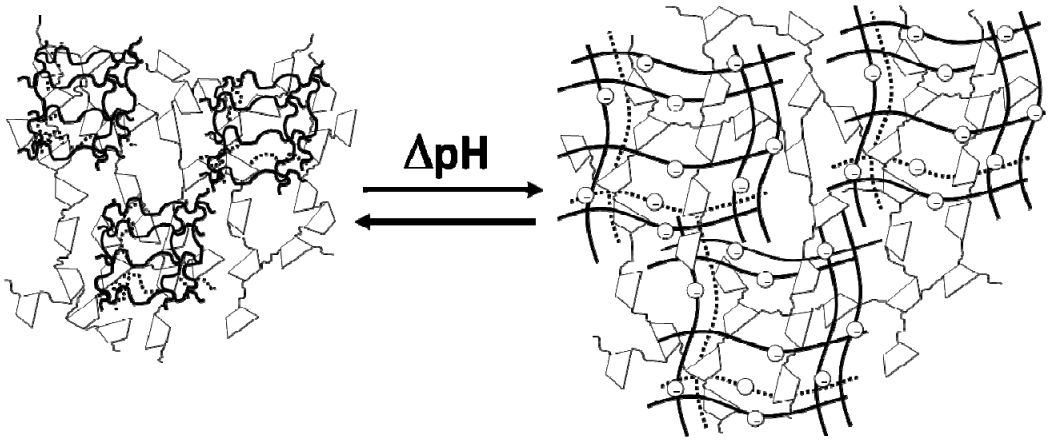

Fig. 1.15. Hinchamiento de un ms-IPN de HP-β-CD y carbopol en respuesta a cambios de pH (Rodriguez-Tenreiro y col., 2007b).

Los ms-IPNs han mostrado un gran potencial como vehículos de estradiol y ketaconazol (*Rodriguez-Tenreiro y col., 2007b*). Un aumento en la proporción de carbopol de 0.2 a 1 %p/p dio lugar a una disminución de la dureza (de 3.0 a 0.7 N), la compresibilidad (de 2.5 a 0.5 N) y el módulo de deformación (de 14 a 2.1 kPa), y a un aumento en la fuerza de bioadhesión (de 0.14 a 0.70 Nmm^{-1}) de los hidrogeles. Los ms-IPNs cargaron más fármaco (hasta 200 veces) que el que se puede disolver en la fase acuosa. La presencia de carbopol contribuyó a incrementar la capacidad de incorporación de fármaco al dar lugar, a pH neutro, a entramados de malla más abierta, a través de los que el fármaco difunde con mayor facilidad y puede entrar en contacto más fácilmente con las CDs para formar complejos. Estos IPNs proporcionaron perfiles de liberación sostenida durante varios días, con velocidad de cesión sensible al pH y modulable modificando la proporción de carbopol (Fig. 1.16).

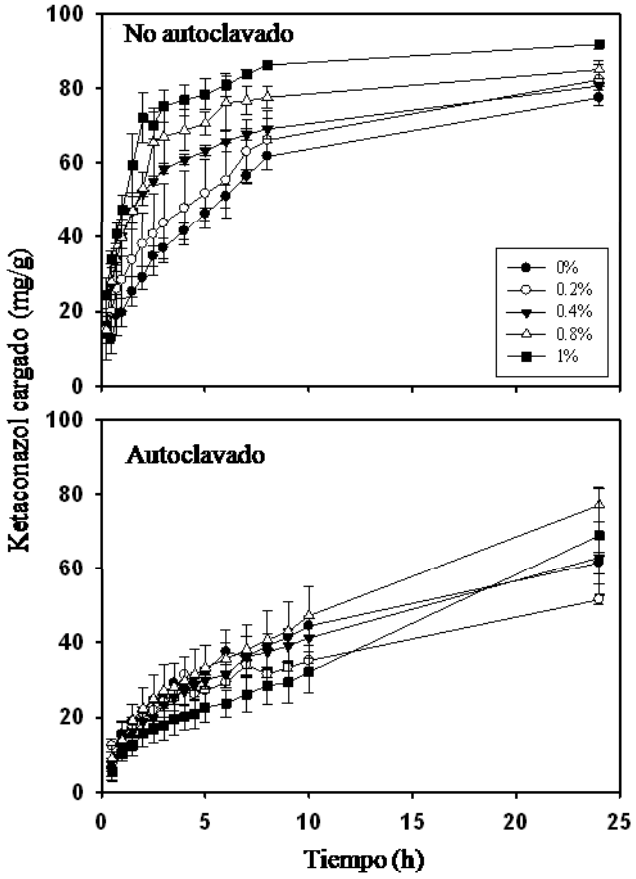

Fig. 1.16. Perfiles de cesión de ketoconazol, en una disolución de dodecilsulfato sódico al 0.3% (pH 7.8), a partir de hidrogeles HP-β-CD/carbopol preparados con distintas proporciones de carbopol (Rodriguez-Tenreiro y col., 2007b).

b) Hidrogeles preparados por copolimerización de monómeros de CD

Desde hace algunos años los hidrogeles acrílicos se vienen utilizando como componentes de productos sanitarios debido a la versatilidad de sus propiedades mecánicas ya que, por su carácter hidrofílico, permiten que el oxígeno y los nutrientes de pequeño tamaño molecular difundan a su través.

Desde el punto de vista de su aplicación como sistemas de liberación de medicamentos, tienen el inconveniente de que cargan mal fármacos hidrofóbicos y de que ceden rápidamente los fármacos hidrofílicos. La incorporación de CDs al entramado permite superar estas dos limitaciones. Este enfoque requiere la formación previa de monómeros de CD que sean capaces de copolimerizar con los monómeros que entran a formar parte habitualmente de los hidrogeles acrílicos (Fig. 1.17).

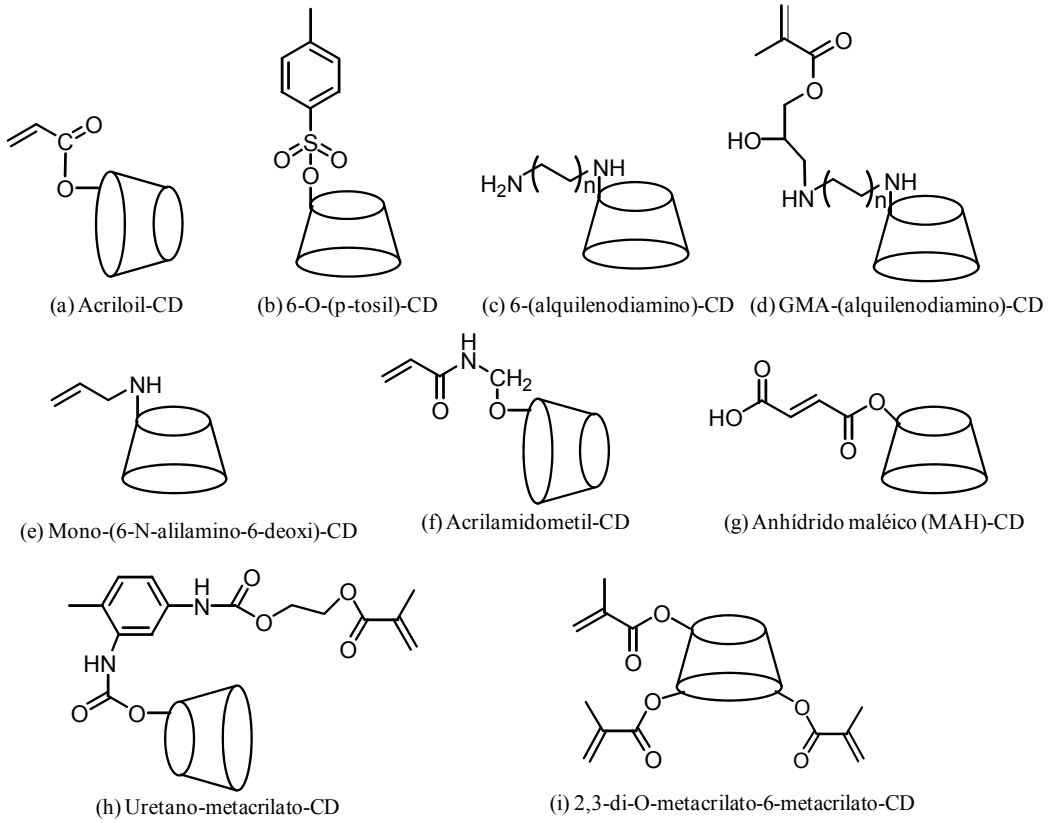

Fig. 1.17. Estructura de los monómeros de ciclodextrina ensayados como componentes de entramados poliméricos.

Se han puesto a punto varias rutas para la síntesis de monómeros de CD. La mayoría de las modificaciones estructurales se centran en los grupos hidroxilo del anillo glucopiranósico. Teniendo en cuenta que las CDs presentan 18 (α-CD), 21 (β-CD) ó 24 (γ-CD) grupos hidroxilo susceptibles de ser sustituidos, el número de derivados posibles es muy elevado. El número total de grupos hidroxilo sustituibles se mantiene en los derivados de CD hidroxialquilados, pero se reduce cuando el sustituyente no es de esta naturaleza. Los grupos hidroxilo en posición C6 son los más nucleófilos. Los que ocupan la posición C2 son los más ácidos y los ubicados en C3 son prácticamente inaccesibles (*Szejtli, 1998*). El hecho de que un elevado número de grupos tenga una reactividad similar, hace que la preparación de monómeros monofuncionalizados resulte difícil. Consecuentemente, en la gran mayoría de las publicaciones se describen monómeros multifuncionales.

En las primeras tentativas de preparar monómeros monofuncionalizados se hizo reaccionar α-CD o β-CD con un éster de m-nitrofenilo en medio alcalino, durante 5 minutos a temperatura ambiente (Fig. 1.17a). Los ésteres de nitrofenilo forman complejos con las CDs y provocan una transesterificación selectiva de uno de los grupos hidroxilo secundarios, reduciendo las posibilidades de formar derivados multifuncionales (*Harada y col., 1976a*). La homopolimerización de acriloil y N-acriloil-6-aminocaproil monómeros de CD o su heteropolimerización con otros monómeros hidrosolubles, condujo a la formación de polímeros solubles en agua (*Harada y col., 1976a*). La poliacriloil-β-CD presenta una menor afinidad que la β-CD por moléculas de pequeño tamaño, como el ácido m-clorobenzoico y el ácido cinámico; en cambio, la afinidad por moléculas con dos anillos aromáticos, como el rojo metilo y el naranja I, es mayor. Estos resultados sugieren que las unidades de β-CD que forman parte de las cadenas del polímero actúan cooperativamente para atrapar moléculas de gran tamaño (*Harada y col., 1976b*).

La copolimerización de acriloil-β-CD con N-isopropilacrilamida (NIPA) da lugar a hidrogeles porosos que experimentan rápidas transiciones de fase en medio acuoso (*Zhang y col., 2004b*). El grupo de Asanuma y Komiyama estudió con detalle la preparación de entramados imprinted para moléculas capaces de formar complejos en los que participan varias unidades de CD. La acriloil-α-CD y la acriloil-(6-O-α-D-glucosil)-β-CD se polimerizaron en presencia de diferentes moléculas diana (vancomicina, cefazolina, feneticilina, y algunos dipéptidos) para obtener partículas rígidas con una microestructura capaz de ajustarse a las características de cada molécula diana (*Asanuma y col., 2001*). Por término medio, los entramados imprinted cargaron el doble de fármaco que los entramados preparados en ausencia de las moléculas diana. Piletsky y col. observaron que cuando se combinan monómeros de bisacriloil-β-CD con monómeros funcionales capaces de interaccionar electrostáticamente (ácido sulfónico 2-acriloilamido-2,2´-dimetilpropano) se obtienen entramados con gran afinidad por moléculas anfifílicas como la fenilalanina, discriminando incluso sus enantiómeros (*Piletsky y col., 1999; Piletsky y col., 2005*).

Los derivados monotosilo de la β-CD se pueden obtener haciendo reaccionar un grupo hidroxilo C6 primario con el cloruro de tosilo (Fig. 1.17b) (*Seo y col., 1987*). El precursor 6-Ots-β-CD se emplea para funcionalizar la polivinilamina que se usa en cromatografáa (*Crini y col., 1997*) y para mejorar las prestaciones de polímeros naturales como vehículos de fármacos (*Ramirez y col., 2006*). Para preparar nuevos monómeros monofuncionalizados con grupos amino primario, se hace reaccionar etilenodiamina (EDA) o 1,6-hexanodiamina (HAD) con mono-6-Ots-β-CD para obtener β-CD (*Liu y col., 2003*). A continuación, el grupo amino reacciona con glicidilmetacrilato (GMA) para formar GMA-EDA-β-CD y GMA-HAD-β-CD, que son monómeros monometacrilato de β-CD (Fig. 1.18).

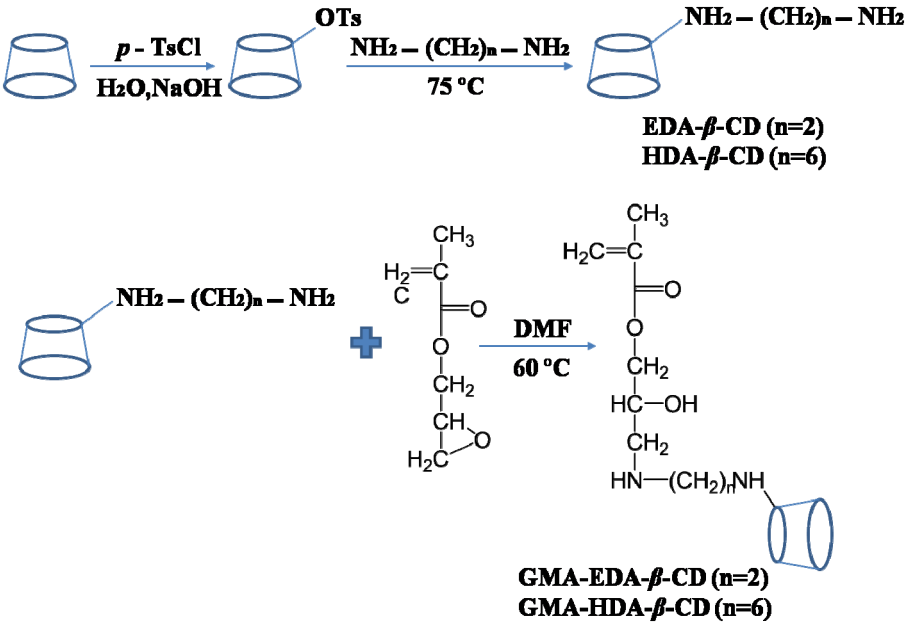

Fig. 1.18. Ruta de síntesis de monómeros monometacrilato de β-CD (GMA-EDA-β-CD y GMA-HDA-β-CD) a partir de mono-6-OTs-β-CD y etilenodiamina (EDA) ó 1,6-hexanediamina (HAD) (Liu y col., 2003).

Los copolímeros de NIPA y monometacrilato-β-CD presentan una elevada solubilidad en agua y mantienen la sensibilidad a la temperatura característica de NIPA. La preparación de copolímeros lineales por polimerización de NIPA con GMA y posterior conjugación con el derivado etilenodiamino de mono-6-Ots-β-CD se ve dificultada por la lentitud con la que transcurre la conjugación y por la rápida reticulación de las cadenas del GMA-NIPA durante el proceso de secado (*Liu y col., 2005*). La copolimerización de 2-hidroxietilacrilato (HEA; 87-100 mol%) con GMA-EDA-β-CD (0-13 mol%) es también una reacción de bajo rendimiento debido a que la homopolimerización del HEA es más rápida que la copolimerización, y se obtienen hidrogeles con un contenido en CD mucho menor que el esperado. La GMA-EDA-β-CD causa un incremento marcado en la

temperatura de transición vítrea, reduce la capacidad de hinchamiento y retrasa la liberación de melatonina (de 90% a 70% a los 120 minutos) (*Liu y Fan, 2005*).

Los monómeros derivados de la acrilamida, como la acrilamidometil-CD (Fig 1.17f), se pueden preparar fácilmente haciendo reaccionar N-metilolacrilamida con la CD (*Lee y col., 2001*). Este proceso transcurre en medio ácido y usa acetona como único disolvente orgánico. La NMA-β-CD con 1-3 grupos acrilamidometilo por molécula de CD se ha utilizado para funcionalizar fibras de algodón (*Lee y col., 2001*). Por este mismo procedimiento, se sintetizó NMA-γ-CD monosustituida, que se copolimerizó con acrilato sódico (3-4 M) para obtener hidrogeles sensibles a cambios de pH que se cargan con fármacos que forman complejos de inclusión. El monómero de NMA-γ-CD es muy soluble agua y se puede polimerizar por radicales libres en medio acuoso utilizando BIS (13-39 mM) como agente reticulante (*Siemoneit y col., 2006*). Independientemente de la proporción de NMA-γ-CD, se obtuvieron hidrogeles que hinchados son muy flexibles y transparentes. Hidrogeles de composición similar preparados sin NMA-γ-CD cargaron cantidades muy bajas de triamcinolona, debido a la pobre afinidad del fármaco por los componentes del hidrogel y al bajo grado de hinchamiento en el medio etanol/agua usado para la carga. Cuando se sintetizaron hidrogeles con cantidades de NMA-γ-CD de 15 mg/ml o superiores, la capacidad de carga y el hinchamiento se incrementaron considerablemente. Los hidrogeles de NMA-γ-CD fueron capaces de liberar tramcinolona durante 24 horas independientemente del pH del medio, lo que indica que el proceso de cesión está regulado por la afinidad del fármaco por la CD. En el caso del propranolol, la carga máxima se observó con los hidrogeles sintetizados sin NMA-γ-CD debido al descenso en la proporción de grupos ácido que se produce a medida que aumenta la cantidad de NMA-γ-CD. La cesión del propranolol, de manera similar a la de la triamcinolona, tampoco se vio afectada por las variaciones en el grado

de hinchamiento derivadas de los cambios de pH. De hecho, la liberación resultó incluso más lenta a pH 7.4, medio en el que los hidrogeles están totalmente hinchados, lo que confirma el importante papel de las interacciones fármaco-entramado polimérico en el control de la cesión.

La posición de los grupos acrilamida en el anillo de glucopiranosa resulta determinante para la funcionalidad del entramado. Por ejemplo, monómeros de acrilamida-β-CD con un doble enlace reactivo en la parte ancha o en la zona estrecha del cono truncado de la CD, dan lugar a entramados capaces de captar aminoácidos u oligopéptidos en agua (*Osawa y col., 2006*). Las moléculas diana se incorporaron a la mezcla de reacción para promover un reordenamiento de los monómeros de CD que haga posible la obtención de hidrogeles con capacidad de reconocimiento selectivo de las moléculas diana. Una vez que se orientan las moléculas susceptibles de ser alojadas en la cavidad de la CD, la posición del doble enlace reactivo en el monómero de CD determina la distancia entre la molécula diana y el grupo polimerizable. La mayoría de las moléculas que forman complejos con la β-CD se introducen por la parte más ancha del anillo (este es el caso de la N-benziloxicarboniltirosina en Fig. 1.19) independientemente de la posición del grupo vinilo (*Bender y Komiyama, 1978*). La mono-3-(N-acrilamido)-3-deoxi-altro-β-CD (3-AAm-CD) y la mono-6-(N-acrilamido)-6-deoxi-β-CD (6-AAm-CD) dan lugar a entramados con diferente microestructura (Fig. 1.19). El 3-AAm-CD da lugar a cavidades receptoras pequeñas y adaptadas a la forma de la N-benciloxicarboniltirosina. En consecuencia, los entramados de 3-AAm-CD reconocen selectivamente la N-benciloxicarboniltirosina y tienen una baja tendencia a captar otros aminoácidos y péptidos de mayor tamaño. Las cavidades de mayor tamaño a las que da lugar el 6-AAm-CD, que presenta el grupo vinilo hacia el lado opuesto de la molécula diana, son menos selectivas (*Osawa y col., 2006*).

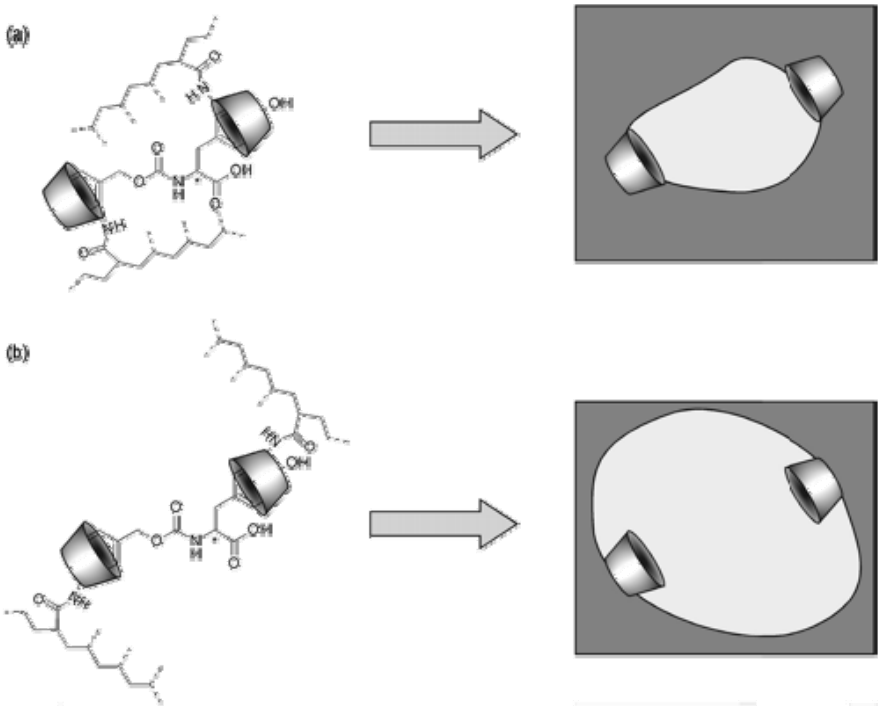

Fig. 1.19. Distribución espacial del mono-3-(N-acrilamido)-3-deoxi-altro-β-CD (a) o mono-6-(N-acrilamido)-6-deoxi-β-CD (b) durante la polimerización y la reticulación con N,N′-metilenobis(acrilamida) en presencia de N-benziloxicarboniltirosina (Osawa y col., 2006).

El 6-AAm-CD se ha mostrado muy útil para preparar entramados con receptores artificiales capaces de diferenciar angiotensina I de angiotensina II, dos oligopéptidos con secuencias de aminoácidos muy similares. Los excelentes resultados obtenidos cuando se usó este material como fase estacionaria de HPLC prueban que el efecto imprinting está relacionado con la conformación del oliogopéptido en medio acuoso más que con su estructura primaria (*Song y col., 2007*).

La condensación de CDs con anhidrido maleico (MAH) conduce a la obtención de monómeros que combinan la capacidad complejante de las CDs con la capacidad de respuesta frente a cambios de pH de los grupos carboxílicos (Fig. 1.17g). El número de grupos vinilo y ácido carboxílico por unidad de CD se puede modular controlando la relación molar de MAH:β-CD (*Liu y Fan, 2002*). Los hidrogeles de PNIPA(91-64% p/p)-co-MAH-β-CD(9-36% p/p) responden a estímulos térmicos, pH y fuerza iónica y se puede utilizar para cargar clorambucilo y cederlo en función del pH del medio (*Liu y col., 2004c*). Los cambios en el grado de hinchamiento son reversibles y reproducibles después de varios ciclos, lo que prueba su verdadero carácter inteligente (*Liu y Fan, 2002*) (Fig. 1.20). También se pueden obtener hidrogeles sensibles al pH y a la fuerza iónica irradiando disoluciones acuosas de MAH-β-CD:ácido acrílico (AA) con un haz de electrones (*Shan y col., 2009*). Los hidrogeles se contraen a un pH comprendido entre 1 y 3 e hinchan considerablemente a un pH comprendido entre 3 y 7, sobre todo en medios de fuerza iónica baja.

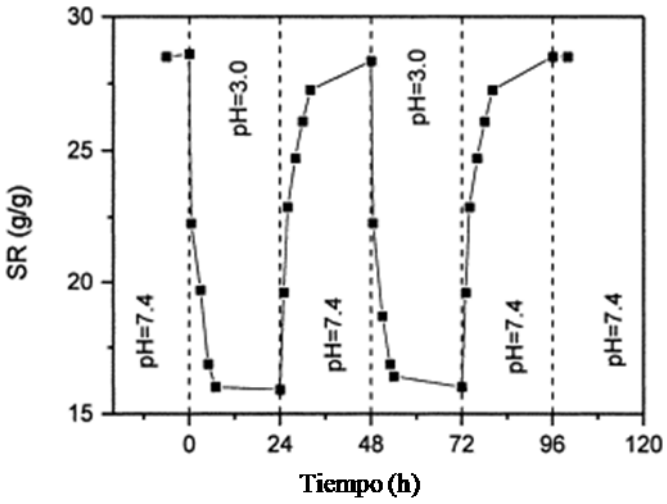

Fig. 1.20. Respuesta al pH de hidrogeles PNIPA-co-MAH-β-CD (Liu y Fan, 2002).

La MAH-β-CD también se puede copolimerizar con macromonómeros de Pluronic® F68 y poli(ϵ-caprolactona). Los Pluronic®, un grupo de copolímeros bloque de poli(oxido de etileno)-poli(oxido de propileno)-poli(oxido de etileno) (PEO-PPO-PEO), sufren transiciones sol-gel que son sensibles a cambios de temperatura (*Kabanov y col., 2002*). Las dispersiones de Pluronic que gelifican a 37ºC son muy útiles para la formación *in situ* de sistemas depot, aunque tienen el inconveniente de que las propiedades mecánicas de los geles son deficientes (*Sosnik y col., 2003; Cohn y col., 2006*). Se puede inducir la copolimerización de monómeros acriloilo de Pluronic F68-g-poli(ϵ-caprolactona) (5-30%) con MAH-β-CD (70%), que actúa como agente reticulante, irradiando con luz ultravioleta. La proporción de MAH-β-CD permite ajustar las propiedades mecánicas del hidrogel (*Ma y col., 2008*). Proporciones altas de MAH-β-CD conducen a valores elevados de los módulos de carga (G´) y de pérdida (G´´). La capacidad de estos hidrogeles para cargar fármaco y controlar su cesión aun no se ha ensayado. Con el fin de obtener entramados biodegradables se copolimerizó un macromonómero de poli(D,L-ácido láctico) (PLA) con una β-CD polimerizable (obtenidos ambos por reacción con 1-alliloxi-2,3-epoxipropano) en dimetilsulfoxido/tolueno a 70ºC. La velocidad de degradación de los hidrogeles en tampón fosfato a 37ºC resultó ser dependiente del contenido en monómero de β-CD y del número de dobles enlaces reactivos (*Lu y col., 2008*).

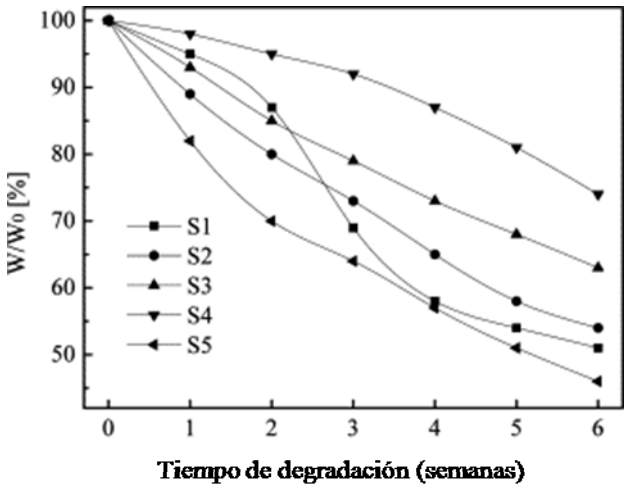

Fig. 1.21. Influencia del grado de reticulación y del número de grupos vinilo presentes en la CD (1.2 en S4, 3.1 en S1 a S3 y 6.7 en S5) en la velocidad de degradación de hidrogel de PLA:βCD preparados con distintas relaciones molares (S1=1:3; S2=S4=S5=1:1; S3=3:1) (Lu y col., 2008).

Para preparar monómeros multifuncionales de uretano-metacrilato-β-CD (Fig. 1.17h) se suele acudir a un procedimiento en dos etapas: a) se sintetiza un derivado de uretano-metacrilato a partir de hidroxietilmetacrilato (HEMA) y tolueno-2,4-diisocianato, y b) este derivado se hace reaccionar con β-CD (*Demir y col., 2008*). La reacción de uretano-metacrilato-β-CD (0-2.5mol%) con HEMA (87.5-90 mol%) y el agente reticulante el poli(etilenglicol)diacrilato conduce a la formación de un hidrogel. La incorporación del monómero de β-CD al 2.5mol% hace que el grado de hinchamiento del hidrogel pase del 34 al 50% e incrementa la capacidad de carga de ácido salicílico, sulfatiazol, rifampicina y naranja de metilo. El efecto sobre la velocidad de liberación resultó ser dependiente de la hidrofilia del fármaco, ralentizándose ligeramente la liberación de naranja de metileno y ácido salicílico, y acelerándose la del fármaco hidrofóbico sulfatiazol

(Fig. 1.22). Las diferencias en la solubilidad y en la afinidad de los fármacos por las CDs explican los efectos contrapuestos que se derivan de la incorporación del monómero al hidrogel.

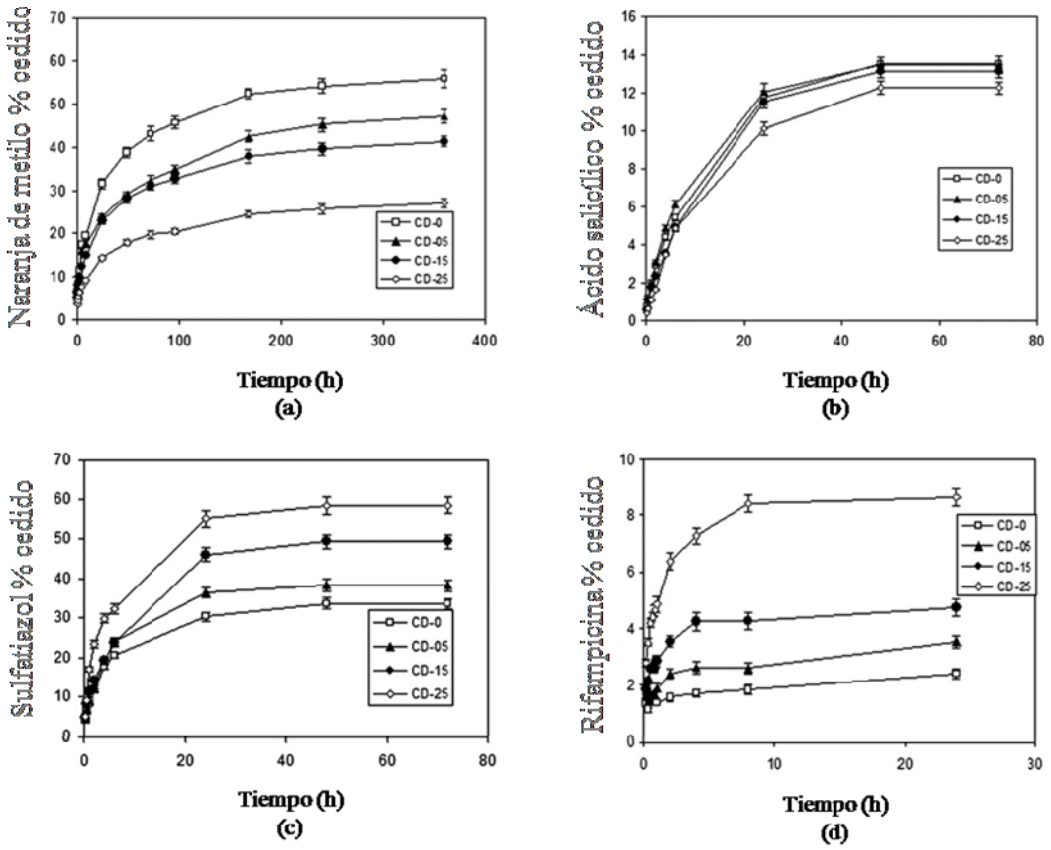

Fig. 1.22. Perfiles de liberación de (a) naranja metilo, (b) ácido salicílico, (c) sulfatiazol y (d) rifampicina a partir de hidrogeles de β-CD-UM (Demir y col., 2008).

Los monómeros metacrílicos de CDs despiertan un gran interés por sus potenciales aplicaciones en una gran variedad de campos, hasta tal punto que el 2-hidroxi-3-metacriloiloxi-propil-β-CD (βW7MAHP) con una media de dobles enlaces de 2.5 por unidad de HP-β-CD llegó a estar comercializado (Wacker-

Chemie GmbH). Este monómero se preparaba haciendo reaccionar glicidilmetacrilato (GMA) con HP-β-CD en medio alcalino (Janus y col., 2003). Los entramados de βW7MAHP y sus copolímeros con 2-hidroxietilmetacrilato (HEMA) se mostraron útiles para captar contaminantes del agua (*Janus y col., 1999*). La copolimerización con HEMA dio lugar a notables incrementos en el grado de hinchamiento en agua y mejoró la capacidad de sorción de los entramados de βW7MAHP al facilitar el acceso hacia las cavidades de las CDs. La copolimerización de βW7MAHP con 1-vinil-2-pirrolidona permite obtener polímeros solubles (proporción de βW7MAHP inferior al 60 mol%) y también hidrogeles reticulados (proporción de βW7MAHP mayor que el 60%) (*Janus y col., 2003*).

También se pueden obtener monómeros de CDs con 6 dobles enlaces en una sola etapa haciendo reaccionar β-CD con anhídrido metacrílico empleando hidróxido sódico como catalizador (*Zawko y Schmidt, 2006*). La fotopolimerización directa de β-CD metacrilada en disolución al 6% conduce a la formación de hidrogeles, mientras que a concentraciones superiores al 8% se generan entramados frágiles. Los monómeros de CD con grupos metacrílicos en las posiciones 2 y 3 se pueden obtener por acetilación del grupo hidroxilo primario y esterificación del grupo hidroxilo secundario con anhídrido metacrílico (*Saito y col., 2001*). Estos monómeros de CD se han empleado con éxito como moldes de polimerización para obtener polímeros metacrílicos de 10 a 14 unidades (*Saito y col., 2002*). La esterificación completa de los grupos hidroxilo primarios (7) y secundarios (14) permite preparar el monómero (2,3-di-O-metacrilato-6-metacrilato)-β-CD (*Saito y col., 2003*) (Fig. 1.23). La complejación del monómero de β-CD metacrilada con moléculas huésped modifica notablemente la conformación espacial de los dobles enlaces reactivos y, en

consecuencia, la capacidad del monómero para actuar como molde de polimerización de otros monómeros (*Saito y col., 2005*).

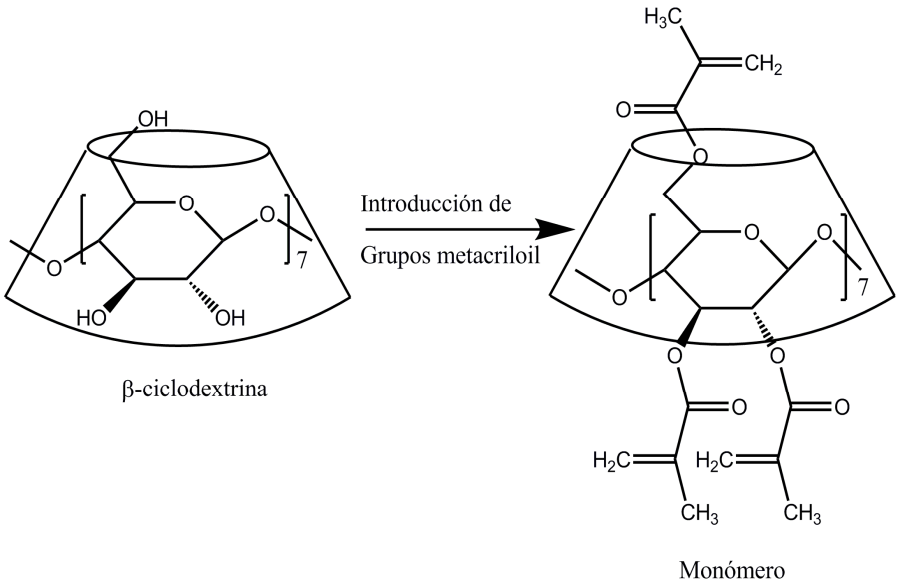

Fig. 1.23. Esterificación completa de los grupos hidroxilo primarios y secundarios para obtener el monómero (2,3-di-O-metacrilato-6-metacrilato)-β-CD (Saito y col., 2003).

Los monómeros de metacrilato también se pueden utilizar para preparar rellenos para empastes dentales. La incorporación de fotoiniciadores, como la camforquinona y el etil-4-dimetilaminobenzoato, resulta en la formación de complejos de inclusión con los monómeros, alterando sustancialmente la fuerza de adhesión y el grado de conversión durante la preparación del empaste (*Hussain y col., 2004; Hussain y col., 2005a*). Ajustando la relación grupos metacrilato polimerizables/ grupos hidroxilo se pueden obtener monómeros de CD adhesivos que promueven el anclaje del empaste a la dentina. Las resinas preparadas con un 33% de β-CD metacrilada, 30% HEMA y 37% acetona muestran una resistencia a

la fuerza de cizalla (16 MPa) similar a la de otros empastes comercializados (*Hussain y col., 2005b*).

1.4. Funcionalización con CDs de entramados preformados

La funcionalización con CDs de materiales preformados es un área en la que se está desarrollando una investigación cada vez más intensa. Los biomateriales deben combinar propiedades estructurales y superficiales aptas para una determinada aplicación. Ciertas propiedades estructurales, como la resistencia física y la estabilidad química, determinan la duración del material, mientras que las características superficiales condicionan la naturaleza y la intensidad de las interacciones cuando el material entra en contacto con otros materiales o con los tejidos vivos. La funcionalización de superficies con CDs abre un abanico de posibilidades a la hora de modular la afinidad de la superficie (en especial cuando son altamente hidrofóbicas) por determinadas moléculas. Por ejemplo, se pueden anclar CDs a materiales textiles para que retengan colores, esencias, repelentes de insectos o, incluso, sustancias con actividades antimicrobianas (*Wang y col., 2004; Romi y col., 2005; Hebeish y col., 2008*). La velocidad de volatilización de las esencias que impregnan el algodón con CDs inmovilizadas es muy lenta, y la resistencia al lavado muy alta (*Wang, y Chen, 2006*). La modificación superficial de materiales que se utilizan en la fabricación de dispositivos médicos con CDs conduce a una reducción en la adsorción de proteínas y a una mayor hemocompatibilidad (*Zhao y col., 2007*).

En el caso particular de los hidrogeles, con la funcionalización final con CDs se busca dotarlos de capacidad de formación de complejos manteniendo las propiedades estructurales de los entramados. Como se mencionó anteriormente, los monómeros de CD suelen presentar varios dobles enlaces reactivos, de manera que cuando se incorporan a la estructura del hidrogel actúan como agentes

reticulantes que modifican sus propiedades viscoelásticas, mecánicas y de hinchamiento. El anclaje de CDs a hidrogeles preformados permite dotarlos de nuevas funcionalidades manteniendo las propiedades estructurales del entramado.

Se han diseñado hidrogeles capaces de experimentar de forma autónoma transiciones de volumen (ciclos de hinchamiento/contracción) en los que β-CD actúa como sensor de una sustancia y NIPA como actuador (*Ohashi y col., 2006*). Para ello, se copolimerizó NIPA (20 g) con p-nitrofenolacrilato (3.4 g) en N,N´-dimetilformamida y, a continuación, se incorporaron CDs aminadas a los grupos p-nitrofenilacrilato (Fig. 1.24).

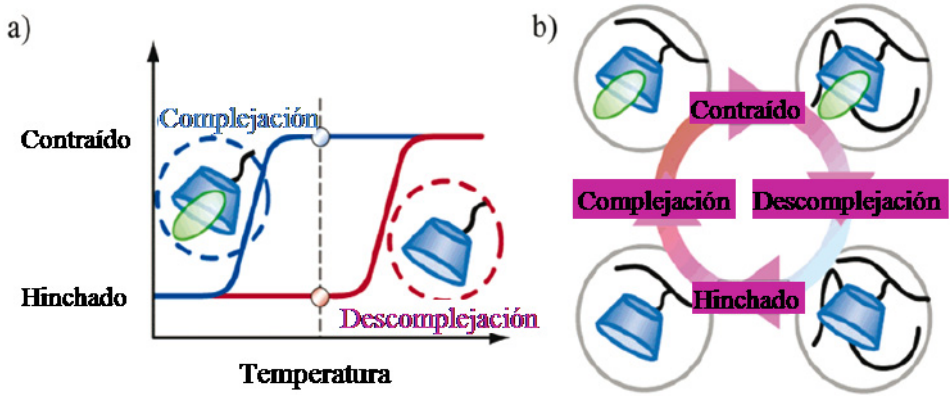

Fig. 1.24. (a) Cambios de volumen inducidos por modificaciones en la temperatura de un hidrogel de poli(NIPA-co-CD) en presencia de 8-anilino-1-naftaleno-sulfónico, y (b) esquema del fenómeno oscilatorio autónomo: contracción del polímero, decomplejación del 8-anilino-1-naftaleno-sulfónico, hinchamiento del polímero y formación del complejo con el 8-anilino-1-naftaleno-sulfónico (Ohashi y col., 2006).

La complejación de la CD con la molécula huésped (ácido 8-anilino-1-naftaleno-sulfónico) altera el equilibrio hidrofílico/hidrofóbico, reduce la temperatura de transición y hace posible que se contraiga el entramado polimérico (Fig. 1.24a). La contracción desestabiliza el complejo provocando la

decomplejación, con lo que se restablece la temperatura de transición y el hidrogel se hincha. La coordinación de estos dos efectos (complejación/contracción) a una temperatura intermedia entre la de transición del hidrogel cuando las CDs están formando complejos, y la de transición cuando las CDs se encuentran libres, determina que el hidrogel experimente cambios autónomos de volumen (Fig. 1.24b). Estos hidrogeles presentan un elevado potencial como sensores.

2. Planteamiento y objetivos

2. PLANTEAMIENTO y OBJETIVOS

El presente trabajo se ha planteado con el objetivo de mejorar la capacidad de los hidrogeles de polihidroxietilmetacrilato-de uso habitual en el campo de la biomedicina como componentes de lentes de contacto e implantes-para cargar fármacos y regular la cesión, incorporando ciclodextrinas al entramado polimérico. Se pretende combinar en un mismo entramado las propiedades de los hidrogeles acrílicos, con la capacidad de las ciclodextrinas para formar complejos de inclusión. Para ello se han abordado las aproximaciones siguientes:

a) Síntesis de monómeros de ciclodextrina, y posterior copolimerización con monómeros acrílicos.
b) Anclaje de ciclodextrinas en hidrogeles preformados, aplicando procedimientos que no requieran modificaciones previas de la estructura de la ciclodextrina y que no causen cambios en las propiedades físicas y mecánicas de los hidrogeles acrílicos de partida.

Los hidrogeles funcionalizados que se desarrollen por medio de estas dos aproximaciones serán una caracterizadas en profundidad en lo que se refiere a

rendimiento de incorporación de ciclodextrinas y propiedades determinantes de su aplicabilidad como biomateriales, en particular como lentes de contacto medicadas. Se prestará atención a las propiedades superficiales, mecánicas y de hinchamiento, a la claridad óptica y a la citocompatibilidad. También se evaluará la capacidad de incorporación y de control de la cesión de fármacos, principalmente antiinflamatorios y antimicrobianos, que pueden ser de interés para el desarrollo de productos sanitarios medicados.

3. Publicaciones y Patente

Hydrogels with cyclodextrins as highly versatile drug delivery systems.

En: Handbook of Hydrogels: Properties, Preparation & Applications. Stein, D.B. (ed), Nova Science Publishers, Inc., New York 2009, Chapter 3.

In: Handbook of Hydrogels: Properties, Preparation... ISBN: 978-1-60741-702-6
Editor: David B. Stein

Chapter 3

HYDROGELS WITH CYCLODEXTRINS AS HIGHLY VERSATILE DRUG DELIVERY SYSTEMS

Carmen Alvarez-Lorenzo,[1]* Fernando Rosa dos Santos,[1] Alejandro Sosnik,[2,3] Juan J. Torres-Labandeira,[1] and Angel Concheiro[1]

[1]Departamento de Farmacia y Tecnologia Farmaceutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain.
[2]Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires and
[3]National Science Research Council (CONICET), 11113-Buenos Aires, Argentina

ABSTRACT

Cyclodextrins (CDs) are cyclic oligosaccharides that combine a hydrophobic cavity with a hydrophilic surface. This unique characteristic confers the CDs the ability to form inclusion complexes with a wide range of drugs. Their complexation ability has been widely exploited as a mean to increase the solubility and stability of drugs in liquid formulations or to promote drug dissolution from solid formulations. CD-functionalized materials may open new perspectives in phamacotherapy. Combination of the features of CDs and hydrogels in a single hybrid material is particularly attractive. Hydrogels have emerged as very suitable platforms for developing advanced drug delivery systems (DDS) owing to their renowned biocompatibility, fine tunable mechanical properties and versatile composition, which makes them suitable for any delivery route. Nevertheless, their performance as DDS is somehow limited when a high loading of hydrophobic drugs (that dislike the aqueous phase of the hydrogels) or when a precise control of the delivery of hydrophilic drugs is required. CDs can overcome these limitations providing the hydrogels with complexation functionality. The CD cavities can act as suitable reservoirs for hosting poorly-water soluble drugs within the aqueous environment of the network, leading to a remarkable increase in the network/water partition coefficient of the drug. Furthermore, the cross-linked structure leads to a microenvironment rich in CDs that do not disassembly under dilution in the biological fluids. Such highly rich CD domains prevent from a fast drug decomplexation, resulting in a controlled delivery even for

*Corresponding author: Carmen Alvarez-Lorenzo. Fax: 34981547148; e-mail: carmen.alvarez.lorenzo@usc.es

hydrophilic drugs. This chapter analyzes the role of movable CDs in semisolid or solid networks and of fixed CDs in cross-linked networks, and revise the main approaches developed for preparing CD-based hydrogels: i) direct cross-linking of CDs through condensation reactions with di- or multifunctional reagents, ii) free radical copolymerization of CD derivatives with acrylic monomers, and iii) grafting of CDs to preformed hydrogels. Examples of the performance of CD hydrogels as DDS, including stimuli-responsive systems, are presented and their practical applicability discussed.

INTRODUCTION

In parallel with the discovery of new candidates to drugs, the design of delivery systems adapted to the physic-chemical characteristics of the therapeutic substances and capable to transport them towards the site of action and to regulate their release rate as a function of therapeutic demands is attracting much attention [1,2]. One of the earliest problems that a formulator must face up is that most drugs do not have an adequate balance between aqueous solubility and membrane permeability. Such a problem is even more concerning in the case of the novel chemical entities and drug candidates developed *in silico* which are most of them extremely lipophilic [3,4]. New strategies in drug formulation are devoted to overcome the biopharmaceutic limitations of old and novel drugs mainly regarding achievement of adequate concentrations at the absorption place and at the site of action, regulating solubility, permeability, stability, and biodistribution. In this field, cyclodextrins (CDs) are particularly appealing excipients capable of behaving as very versatile tools [5]. Classically used as solubilizers of hydrophobic drugs, CDs can also offer interesting possibilities for regulating the delivery of both hydrophobic and hydrophilic drugs [6].

CDs are cyclic oligosaccharides composed of 6 to 12 D-(+)-glucopyranose units linked by α-(1-4) bonds (Figure 1). Since the discovery of natural α- (6 units), β- (7 units) and γ- (8 units) CDs more than 100 years ago, the production and purification techniques as well as their uses in the pharmaceutical field have substantially evolved [7]. The limited solubility of the natural CDs, in particular β-CD, due to the formation of intramolecular hydrogen bonds, can be overcome substituting some hydroxyl groups of the ring even by hydrophobic moieties. An enormous number of semisynthetic derivatives of CDs have been already synthesized [8,9]. However, only three derivatives of β-CD (Figure 1) together with natural α-, β- and γ-CDs and hydroxypropyl-γ-CD have reached the pharmaceutical grade status. The total degree of substitution [10] of the pharmaceutical grades is about 4.5-7 for 2-hydroxypropyl-β-CD (i.e., 0.65-1.0 hydroxypropyl-moiety per glucose unit), 7 for sulfobutyl-β-CD, and 4-12 for randomly methylated β-CD (i.e., 0.57-1.8 methoxy-moieties per glucose unit) [6]. Nevertheless, not only the solubility but also the dimensions of the CD and the likelihood of forming complexes with other substances are strongly determined by the degree of substitution and, therefore, these variables should be taken into account when choosing a CD for a certain purpose [6,10]. A detalied description of the physico-chemical properties of the CDs can be found elsewhere [6,9,11].

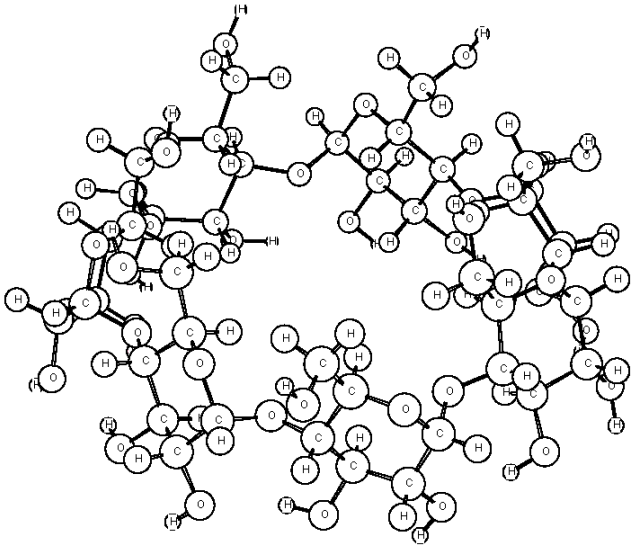

Figure 1. Structure in truncated cone of β-cyclodextrin showing the primary and secondary hydroxyl groups crowning the narrower rim and wider rim, respectively. When some hydrogens of hydroxyl groups at C2, C3 or C6 are replaced by hydroxypropyl groups, methyl groups or sulfobutyl groups derivatives called 2-hydroxypropyl-β-cyclodextrin, methyl-β-cyclodextrin, or sulfobutyl-β-cyclodextrin are obtained.

The truncated cone structure of CDs, with a hydrophilic outer surface and a hydrophobic inner cavity (4.7-8.3 Å diameter, 7.9 Å height), provides them with the capability of forming inclusion complexes with molecules able to completely or partially fit into the cavity. Complexation is commonly seen as the replacement of water molecules, which are energetically unfavored inside the cavity (polar-apolar interaction) with less polar guest molecules. Hydrophobic, electrostatic and van der Waals interactions as well as hydrogen bonding, release of conformational strain, and charge–transfer interaction may be also involved in the complexation process [12]. These relatively weak interaction forces enable hosted molecules within the CDs to be in rapid equilibrium with free molecules in solution. The equilibrium can be shifted towards complexation or decomplexation relatively fast by the addition of cosolvents or certain polymers, a change in pH or an increase in temperature [13-16]. Such complexation capability is been exploited since 1970 in the food industry to mask unpleasant organoleptic features or to stabilize flavoring agents [17]. Furthermore, CD complexes lead to the apparent solubilization of the guest molecule increasing the concentration in aqueous medium up to many orders of magnitude above the solubility coefficient of the guest alone in water [11,18]. Guest-CD complexes in solid state are usually amorphous, which also increases the dissolution rate of the drug. The comprehensive knowledge gained over the years regarding the toxicological aspects of these molecules [19] and the reduction in the cost of the production processes enabled pioneering industries in Japan (1976) and later on in Italy (1988) the commercialization of medicines based on drug-CD complexes in order to solve drug solubility and stability problems [5,20]. The research in this field continues to be very intensive and reviews with comprehensive compilations have been recently published [11,21-23].

Nowadays, there are around 40 medicines based on drug-CD complexes that are world-wide commercialized, mainly for parenteral, ocular and oral routes but also for nasal, topical or sublingual administration, and this number is expected to progressively increase in the next decade [7,11,24]. The reasons behind these optimistic perspectives are mainly based on the capability of CDs to improve drug bioavailability of Class II (low solubility but high permeability) and Class IV (low solubility and permeability) drugs and to move them to Class I in the Biopharmaceutical Classification System (BCS) [25]. The BCS is a binning system for oral drug products that classifies drugs into four groups based on the dissolution yield of a certain dose and on the ability to permeate biologic membranes [26]. This FDA- and EMEA-adopted classification system considers that drugs with optimum solubility and permeability to be orally absorbed belong to Class I [27]. Cyclodextrins and drug-CD complexes are too large (Mw ranging from 1000 to 2000) and hydrophilic to permeate the biological membranes [19]. Thus, only the free drug molecules in equilibrium with the CD-complexes are capable of penetrating the lipophilic membranes. Nevertheless, the dynamic inclusion of lipophilic drugs or of certain lipophilic moieties into the CDs creates a hydrophilic shield that notably increases the apparent drug solubility in the physiological fluids, promoting a fast dissolution of solid drug-CD complexes. Such complexes diffuse easier than the free drug through the aqueous layers at the surface of the mucosa, increasing the number of drug molecules available for permeation at the membrane surface. As the free drug permeates the membrane, a progressive decomplexation occurs in order to replace the absorbed free molecules and to maintain the equilibrium. Furthermore, complexation can increase the stability of the therapeutic substance preventing degradation at the absorption site. These phenomena may enhance drug bioavailability when solubility is the rate-limiting step, as occurs with Class II drugs that easily permeate biological membranes. On the other hand, CDs can act as permeation promoters since they are able to extract lipophilic components from the biomembranes [28] (Figure 2). Drug-CD complexes can also reduce or prevent gastrointestinal, ocular or skin irritation [29], improve organoleptic properties and, if needed, may even provide sustained or targeted drug release [22].

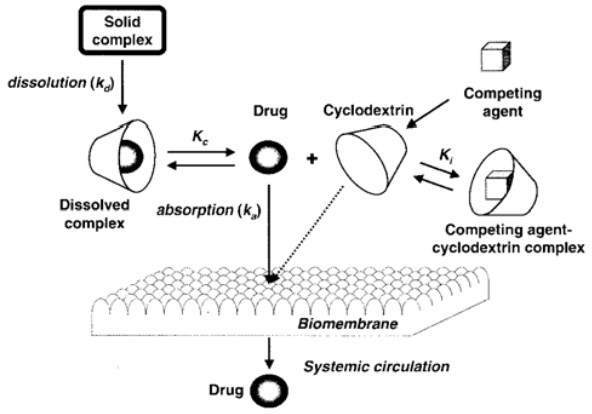

Figure 2. Schematic representation of the dissolution and absorption of a drug from its cyclodextrin complex and competitive binding of membrane components. Reprinted from [22], with permission from the American Chemical Society.

Drug release from common CD complexes, disregarding the strength of the binding constant, is practically instantaneous when the complexes are diluted in aqueous fluids [30-32]. Therefore, in general, CD solutions do not provide a sustained release. Immediate release formulations of cardiac glycosides, analgesics or antiepileptics have been successfully prepared from complexes with hydrophilic CDs. The fast wettability and high solubility of the complexes lead to a rapid oral absorption in emergency situations [33]. Amphiphilic CDs can also self-assembly forming supramacromolecular aggregates and nanospheres that can be loaded with high proportions of hydrophobic drugs (complexed or interacting with complexed drug molecules). Since the drug is molecularly dispersed in the nanostructures, they rapidly release the drug in aqueous medium [34-36]. On the other hand, chemical modification can confer the CDs additional advantageous features. For example, ionizable CDs and hydrophobic CDs can provide delayed and prolonged release, respectively [6]. Cyclodextrins possessing weak carboxylic acid groups show pH-dependent solubility and have been explored for preparing enteric formulations. The 6-O-(carboxymethyl)-O-ethyl-β-CD has been shown particularly useful for this purpose [37]. Drug-CD conjugates may be also potentially useful for developing colon-targeting prodrugs [38]. When slow-release or, preferentially, zero-order kinetics is required, alkylated or acylated CDs can prolong the delivery of water-soluble drugs [39,40]. Despite these remarkable achievements, the capability of CDs alone to control drug release is limited due to a relatively rapid decomplexation when the formulation becomes diluted in the physiological fluids. Since the equilibrium mainly depends on the relative concentration of CD, an approach to control the drug release is to incorporate the drug-CD complexes into structures able to delay such dilution process. Differently from the above described systems formed almost exclusively by drug-CD complexes and that show dissolution-controlled drug release rate, delivery systems made of a network in which the CDs have a restricted mobility can provide diffusion-controlled or affinity-controlled release kinetics. Implementation of other mechanisms is also under study. Accordingly, the next sections will focus on two main strategies pursued to expand the applicability of CDs in drug delivery: i) hydrogels containing physically blended CDs and ii) networks where CDs are covalently bounded to the components of the 3D structure. While in the former, the CDs are less constrained for molecular movement, in the latter they do not move freely due to the covalent binding of the CD to the matrix. Figure 3 depicts the different states in which a CD can be found in a polymeric network.

Hydrogels are outstandingly patient-friendly delivery systems that enable a precise release of drugs for a finite time. Their high biocompatibility makes them convenient drug carriers for being administered using almost any route [41]. Physically cross-linked hydrogels mainly control drug release through the effect of viscosity on drug diffusion. Nevertheless, the hindrance effect is not as high as foreseeable from the apparent macroviscosity, but depends on the viscosity of the microenvironment through which the drug has to pass through [42,43]. Therefore, remarkable increases in macroviscosity of the gel may not provide an effective control of drug release. In contrast, chemically cross-linked hydrogels enable a precise control of drug release by fine tuning the mesh size, which can remain constant or evolve to a smaller or larger size depending on the effect of stimuli or chemical or enzymatic degradation [44]. Their main drawback refers to their limited affinity to most drugs owing to the hydrophilic character, which determines a low loading of hydrophobic drugs or a poor control of the release of hydrophilic drugs. In this context, CDs may overcome some limitations of both physically and chemically cross-linked hydrogels.

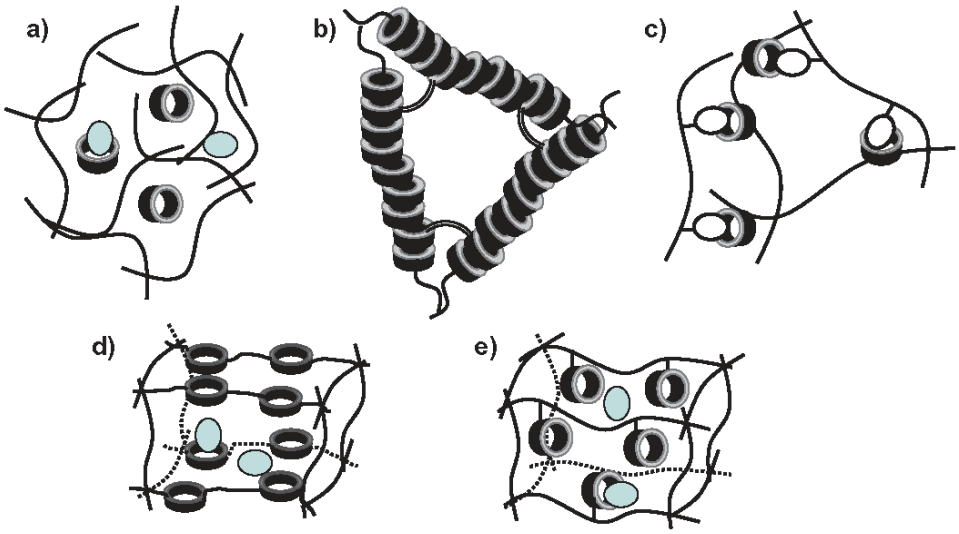

Figure 3. Different states in which a CD can be found in a polymeric network: a) movable CDs; b) poly(pseudo)rotaxanes in which the CDs are chemically bounded ; c) the CDs form part of polymer chains and act as tie-junctions of other polymeric chains; d) the CDs form part of three-dimensional networks; and e) the CDs are hanging from the network structure.

CDs Physically Dispersed in Semisolid or Solid Matrices

Dispersion of CDs in semisolid or solid polymeric matrices (Figure 3a) can significantly modify the drug release rate and thus the drug bioavailability. In general, if the drug is loaded at a concentration above saturation, CDs accelerate the release by enhancing the proportion of diffusible species. Oppositely, when drug concentration is below the solubility limit, complexation reduces the concentration of free drug molecules and hence the release rate [45]. An example of this typical behavior is the enhancement of nasal permeation of melatonin from hydroxypropyl methylcelulose (HPMC 4000 cPs) gels when 2-hydroxypropyl-β-CD (HP-β-CD) or randomly methylated β-CD (M-β-CD) are added at low concentrations (1%), owing to a partial drug solubilization that facilitates the diffusion and the rapid complexation/decomplexation equilibrium. At greater CD concentrations (5-10%), the nasal permeation is significantly diminished since the whole dose is solubilized as stable complexes and the number of free drug molecules available to permeate is minor [46]. Nevertheless, the apparently contradictory results reported [47,48] can be attributed to the concomitance of other effects, mainly steric hindrance, odd dependence of the complexes solubility on certain variables of the medium, and interactions of the drug or the CDs with the polymeric components of the formulation, as explained below.

Complexation enables the formulation of hydrophobic drugs as dissolved solutes in hydrophilic polymer systems [49]. If the drug forms stable complexes with the CDs, diffusion rate inside the matrix diminishes compared to that of the free drug owing to the lower

diffusivity of the bulky complex [50]. The network mesh size becomes a relevant parameter that discriminates diffusible species as a function of their hydrodynamic volume. Several mathematical models have been developed to analyze drug release profiles from swollen polymer networks and to determine the diffusivity of free drug and of drug-CD complexes [51]. Studies carried out with cross-linked polyethyleneglycol revealed that 90% nicardipine dose was delivered in 4 hours. The addition of β-CD in amounts enough to achieve nicardipine:β-CD 1:1, 1:2 or 1:3 molar ratio did not modify the swelling degree of the network, but caused a progressive decrease in release rate; the dose released being 60, 50 or 40% at 4 h, respectively. This finding was due to the fact that complexation with β-CD rendered species of greater hydrodynamic volume and diminished drug diffusivity from $5 \cdot 10^{-8}$ to $1.2 \cdot 10^{-8}$ cm^2/s [51]. Nevertheless, two relevant exceptions to this rule can occur. The first one refers to the case in which drug-CD complexation notably enhances drug solubility and, consequently, facilitates drug dissolution inside swellable polymeric matrices and promotes that the drug and the CD abandon the matrix [52]. Such a greater drug concentration gradient has been shown to facilitate oral and dermal drug absorption [53]. Recently, the effect of HP-β-CD and γ-CD on the release of ibuprofen, ketoprofen and prednisolone from a hydrogel consisting of polyvinylpyrrolidone (PVP) cross-linked with polyethylene glycol-dimethacrylic acid (PEG-DMA) was reported [54]. HP-β-CD and γ-CD differ in size, solubility and affinity towards the drug molecules. Dried hydrogels were alternatively swollen in a small volume of a saturated solution of the drug (control hydrogels) or of a solution of drug:CD complexes. It is important to note that drug:CD molar ratios ranged from 6 to 50 and that the whole dose was solubilized in a medium in which CD was in excess. HP-β-CD increased the amount of drug loaded in the hydrogel by approximately 6, 9 and 3 times for ibuprofen, ketoprofen, and prednisolone, respectively, compared with the load accomplished by the control hydrogels. γ-CD doubled the amount of prednisolone loaded, but it did not enhance the uptake of ibuprofen and ketoprofen due to the lower solubility of the complexes. HP-β-CD remarkably promoted the release of the three drugs, while γ-CD did not alter the release of ibuprofen (due to the low likelihood of complexation) but delayed the delivery of ketoprofen and prednisolone. This last effect was attributed to the lower diffusion coefficient of γ-CD complexes. Other researchers have shown that when the drug-CD affinity constant is sufficiently high, it is not even needed to prepare first the drug-CD complexes, since complexation may spontaneously occur inside the gel layer as matrices made of physical mixtures become hydrated [48].

The second exception is related to drugs that strongly interact with the polymer network. This is the case, for example, of propranolol hydrochloride formulated in poly(acrylic acid) microgels (Carbopol®). The cationic drug forms insoluble ionic complexes with the polymer chains and notably reduces the swelling and the bioadhesion properties of the microgels. Propranolol complexation with β-CD minimizes drug-polymer interactions, restores the behavior characteristic of the microgels and speeds up drug release [55].

If the drug and the CD do not form complexes, hydrosoluble CDs can enhance the release rate by creating channels as the CDs are dissolved and abandon the gel matrix. Oppositely, less hydrophilic CDs can increase the tortuousness of the diffusional path delaying drug release [45]. An example of the intricate and somehow *a priori* unforeseeable role of the CDs on drug release from physically cross-linked networks can be seen on a study carried out incorporating β-CD and HP-β-CD to hydroxypropyl methylcellulose (HPMC K4M) gels and

matrix tablets containing diclofenac sodium (soluble in water) or sulphamethizole (poorly soluble). Both drugs can form complexes with these CDs and the stability constants of diclofenac sodium with β-CD and HP-β-CD have been reported to be 100.6 and 115.2 M^{-1}, respectively, and those of sulfamethizole with β-CD and HP-β-CD 651.8 and 563.9 M^{-1}, respectively [56,57]. The influence of β-CD and HP-β-CD on drug diffusion was particularly evident for gels prepared with a HPMC proportion (2.0%) above its entanglement concentration. In these systems, a drug:CD 1:0.5 molar ratio enhanced the diffusivity preventing the hydrophobic interactions between the polymer and the drug, while a drug:CD 1:3 molar ratio hindered the release. An excess of CD, especially of the bulky HP-β-CD, made the diffusion of the complexes in the relatively low mesh size 2% polymer network more difficult. In the case of matrix tablets, the CDs played an additional role as dissolution promoters. To evaluate to what extent the balance between the increase in dissolution rate and the decrease in diffusion rate determined drug release, matrix tablets were prepared by direct compression of 100 mg drug and 400 mg polymer/CD/lactose blends, whose composition was chosen following a simplex centroid design. A high CD/lactose ratio significantly increased the release rate of hydrophobic drugs (sulphamethizole), but decreased the release rate of hydrophilic drugs (diclofenac sodium), revealing that the predominance of one or other effect depends on the drug hydrophilicity [58].

Finally, partial complexation of the polymeric components with CDs should not be discarded when preparing gels of amphiphilic copolymers or of polymers that bear hydrophobic moieties [59]. For example, CDs can be used to modulate the viscosity and the light-sensitiveness of azobenzene-functionalized polymers. Azobenzene undergoes trans/cis isomerization when irradiated with UV light. Under dark conditions, the hydrophobic trans isomers associate and act as junction points among the polymer chains, resulting in a gel system. Once irradiated, the isomerization prompts the junctions to break since the more hydrophilic cis isomers do not self-associate, which causes the viscosity to decrease. Trans isomers of azobenzene moieties can form complexes with α-CD while the cis isomers can not. Complexation inhibits the self-assembly and may even reverse the effect of UV irradiation on the viscosity of the system [60]. On the other hand, the cis isomer can complexate with HP-β-CD. If the azobenzene moieties belong to an amphiphilic copolymer, such as poly(N,N-dimethylacrylamide-co-methacryloyloxyazobenzene) (DMA-MOAB), addition of HP-β-CD may notably modify, once irradiated at 366 nm, the ability to interact with other amphiphilic copolymers forming mixed micelles. This phenomenon has been shown to significantly alter the diffusion of hydrophilic solutes through the gel system [61].

CDs can also form complexes with block copolymers with a stoichiometry well beyond 1:1 leading to necklace-like supramolecular complexes called polypseudorotaxanes [62]. Polymers possessing hydrophobic segments are threaded by CD molecules which stack along the polymer axis [63-65]. Then, if the polymer ends are conveniently blocked using bulky moieties and the CDs cannot move away the polymer, a polyrotaxane is obtained. Intermolecular interactions between the CD molecules in the polyrotaxanes may result in the formation of superstructures, such as nanorods. It has been also recently proposed to cross-link the CDs of adjacent polyrotaxane chains [66] (see Figure 3 b) or to chemically bond CDs to the end of threadable polymer chains [67] in order to obtain sliding gels. The changes underwent in polymer conformation and performance of the systems make polyrotaxanes an area of growing interest owing to the broad range of biomedical applications that can be

envisioned [62,68,69]. Despite few studies have focused yet on the incidence of polyrotaxane formation on drug solubility and diffusional behavior, the effects have been already shown to be very remarkable [70-72]. Spontaneous complexation of CDs and block copolymers raises the critical micellar concentration of the copolymer and decreases the number of micelles and CD cavities available to host drug molecules. This results in a lower ability to solubilize hydrophobic drugs. Furthermore, in the case of temperature-sensitive polyethylene oxide/polypropylene oxide block copolymers, such as PEO-PPO-PEO poloxamer or Pluronic®, notable changes of the sol-gel transition temperature and of the viscoelasticity of the gel phase may occur [72-74]. Addition of 5 wt.-% HP-β-CD or randomly methylated-β-CD increases by 4-6 ºC or up to 15ºC, respectively, the gelation temperature of 15 wt.-% Pluronic F127 solutions, and significantly decreases the G′ (storage) and G′′ (loss) moduli of the gels. Pluronic F127 unimers can also easily displace host molecules from the CD cavities, increasing the proportion of free molecules in the medium [75]. In summary, these still few studies call our attention on the importance of evaluating the likelihood of complexation and if so, of gaining insight into the nature and stoichiometry of the complexes when preparing drug-polymer-CD ternary gel systems, which is not unusual in pharmaceutical technology.

A further step in this field is the preparation of stable gels using a zipper or key-lock mechanism, in which CDs covalently bounded to a polymer chain recognize certain complexable moieties of other polymers, resulting in a three dimensional network (see Figure 3 c). Such a CD-mediated cross-linking largely increases the viscosity or the gel-like behavior of the system and can be considered as a situation in between physical (i.e., reversible) and chemical (i.e., highly stable upon dilution) cross-linking. This phenomenon has been described in a number of systems made of blends of: i) a polymer bearing pendant β-CD and a polymer with hydrophobic 4-tert-butyl anilide side chains [76]; ii) CD-chitosan conjugates and adamantyl-grafted chitosan or poly(ethylene glycol)s [77]; iii) CD-poly(acrylamide) and poly(acrylamide) possesing aromatic rings [78]; and iv) β-CD polymers (made with epichlorhydrine) and poly(N-isopropylacrylamide) containing either an adamantyl or a dodecyl group [79].

Polymers of β-CD (poly-β-CD) spontaneously self-assemble with dextran chains bearing grafted alkyl side chains (Figure 4). Mixing aqueous solutions of both polymers at 6.6-7.5% w/w led to an instantaneous phase separation. The gel phase was very rich in both polymers and had G′ and G′′ values about 400-500 Pa and 1200-1400 Pa, respectively [80,81]. Lower polymers concentration (0.1-1% w/w) resulted in stable nanogel particles [82]. In addition, polymers bearing ionizable groups formed gels displaying a pH-responsive viscosity profile [83]. The gels were loaded with drugs that formed complexes with poly-βCD before mixing with dextran. Empty cavities (i.e., those that are not occupied by drug molecules) were available to host the alkyl chains and served as junctions between the chains. This type of gels exhibited a sustained delivery of benzophenone and tamoxifen for more than one week. Interestingly the dynamic character of the complexation process enabled the gels to be injected using an 18-gauge needle. After the passage through the syringe, the gels recovered the initial G′ and G′′ values in a few seconds [80]. These features together with an excellent *in vivo* compatibility ensure a promising future for self-assembling CD gels in the biomedical field.

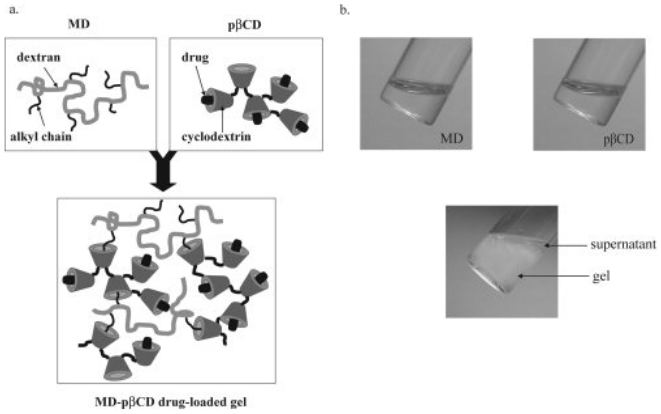

Figure 4. (a) Schematic representation of the spontaneous interaction of alkyl-modified dextran (MD) with poly-β-CD leading to a fast gel formation. Some alkyl chains of the MD are complexed into the CD cavities of the poly-β-CD, leaving also free CDs for the inclusion of hydrophobic drugs. (b) The pictures show the aspect of the MD and poly-β-CD solutions and of the mixture after 5 seconds. Reproduced from [80] with permission of John Wiley & Sons, Inc.

HYDROGELS WITH BUILT-IN CYCLODEXTRINS

Hydrogels in which CDs are forming part of the structure of chemically cross-linked networks can be obtained by direct cross-linking of CDs (condensation with a cross-linker), by copolymerization of CDs with vinyl- or acrylic- comonomers, or by preparing firstly the network and then anchoring the CDs to it. The aim of creating CD networks is to effectively prevent the dilution phenomenon that occurs when the solutions and the physical gels are administered. Since the volume of water that can enter into a chemically cross-linked hydrogel is limited by the own network and the CDs are covalently attached, the hydrogel may swell but the chains do not dissolve and do not move apart. This creates a microenvironment rich in cavities available to interact with the guest drug molecules. In such a network, the drug-CD affinity becomes the main force to retain the drug and to control the delivery. Decomplexation of a drug molecule from one cavity makes the drug available to complexate with a neighbor empty cavity; the likelihood of recomplexation being also dependent on the drug/CD affinity. Therefore, a CD hydrogel can be seen as a network dotted with many dimples and the drug movement can be envisioned as escaping a dimple to fall down another one, which should be abandoned, and so on, up to reach the surface of the hydrogel (Figure 5). The movement of a drug molecule is faster when most dimples are occupied and the likelihood of recomplexation is lower. Oppositely, as the hydrogel delivers the drug, the number of empty CD cavities that are available for hosting the just-passing-through drug molecules increases. Furthermore, some previously released drug molecules could be attracted again towards the network. As a consequence, CD hydrogels possess unique features to be used as sustained release devices. It is important to note that covalent attachment of CDs to a polymeric structure does not decrease their complexation ability but may even improve it, particularly in the case of large molecules that require more than one

CD to fulfil the complexation [84-90]. In advance, the different strategies pursued to develop CD crosslinked networks will be comprehensively reviewed.

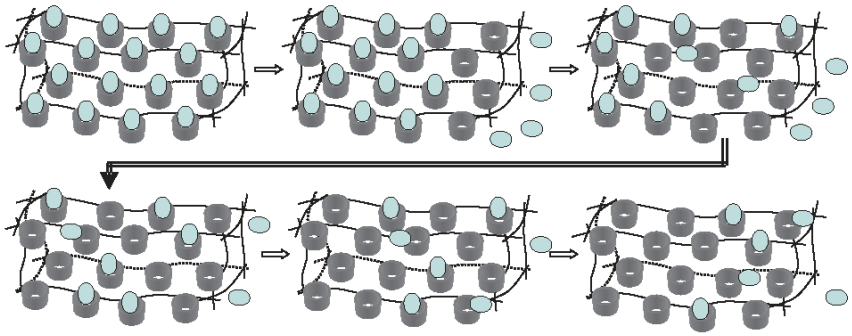

Figure 5. Schematic view of drug release from a chemically cross-linked CD network. The CDs behave as dimples and the drug has to escape the dimples to be released.

Direct Cross-Linking of CDs

First attemps to obtain CD polymers and hydrogels were those based on the condensation reactions of the hydroxyl groups of natural CDs or of the amine or caboxylic acid groups of functionalized CDs with di- or multifunctional cross-linking agents, such as aldehydes, ketones, isocyanates or epoxides (epichlorhydrin or glycilethers) [91]. Despite the condensation is spontaneous, a catalyzer (mainly HO^-, or H^+ in the case of glyoxal or glutaraldehyde [92]) is usually required to speed up the process. The most well-investigated cross-linking agent is epichlorhydrine (EPI). Under alkaline conditions, the two reactive functional groups of EPI can react with the hydroxyl groups of CDs or with other EPI molecules. This results in a mixture of cross-linked CDs joined by repeating glyceryl units of polymerized EPI [93,94] (Figure 6). The EPI-CD hydrogels (usually obtained in the form of microgels) can swell to a large extent in aqueous solutions. A careful control of the reaction process (e.g., quenching the cross-linking process at a certain stage) may lead to hydrosoluble CD polymers [87]. The EPI:β-CD weight ratio also determines the fraction of CD cavities that are available to host guest molecules; the maximum being observed for hydrogels made with 50% β-CD [95].

EPI-CD microgels have been extensively evaluated as adsorbents in the removal process of hydrophobic molecules and drugs from aqueous environments [96-98], as selective traps of food components [99,100] and as fillers of chromatographic columns [101,102], showing a high performance for enantio-resolution of racemic mixtures [103-105]. Comprehensive reviews of the role of CD-based networks in separation science can be found elsewhere [106,107]. For biomedical purposes, a greater hydrophilicity, looser polymer structure and more versatile mechanical properties are usually required. In pionering studies by Szejtli et al. [108] mixed networks of CDs and hydrophilic polymers, such as poly(vinyl alcohol) (PVA), were obtained using EPI and ethylene glycol bis(epoxy propyl)ether as mixed cross-linking agents. Such networks showed faster and higher degree of swelling and improved mechanical

properties, while maintaining the capability of CDs to complexate a wide range of molecules. Later on, the hydrogels were modified with carboxymethyl and acetyl groups, rendering the networks more hydrophobic. The hydrogels showed a high loading capacity of desinfecting drugs, such as ethacridine lactate, brillian green, fuchsin acid, or cetylpiridinium chloride [109].

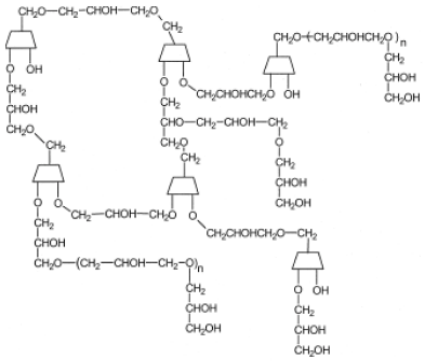

Figure 6. Scheme of a EPI-CD hydrogel. Reproduced from [93] with permission of Wiley-VCH Verlag GmbH &Co.

Combination of EPI-CD with temperature-sensitive poly(N-isopropylacrylamide) (PNIPA) has been explored following two different approaches. As a first strategy, direct grafting of PNIPA to previously cross-linked β-CDs resulted in hydrogels that maintain the transition temperature of PNIPA; being swollen at room temperature and shrunken at 37°C. The use of fluorescent probes revealed that, below the transition temperature, complexation with the cross-linked β-CDs was more favourable than with free β-CDs in solution (ca. 100 times larger affinity constant), which was attributed to the hydrophobic microenvironment that the PNIPA chains provide around the CDs (Figure 7). By contrast, the association constant sharply decreased above the transition temperature due to steric hindrance of the collapsed PNIPA chains, which constrained the access of the fluorescent probe to the CD cavities [110].

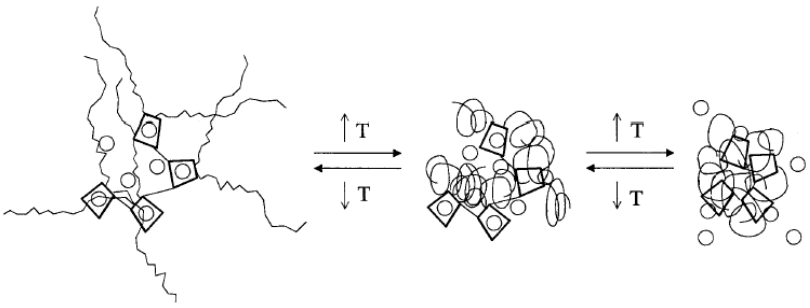

Figure 7. Schematic drawing of the temperature-responsive inclusion of a probe (o) by a cross-linked β-CD network bearing poly(N-isopropylacrylamide) chains. Reproduced from [110] with permission of John Wiley & Sons, Inc.

The second approach consists in preparing interpenetrating (IPN) or semi-interpenetrating (semi-IPN) networks of EPI-CD and PNIPA. For example, a PNIPA hydrogel, cross-linked with N,N'-methylenebis(acrylamide), was synthesized in the presence of a EPI-β-CD network. The IPN maintained the transition temperature of PNIPA but, as opposed to the common PNIPA hydrogels, the swollen IPN was able to control the release of ibuprofen owing to the complexation with the CDs [111]. Similar results were obtained when the release of propranolol from semi-IPNs made of β-CDs grafted to polyethyleneimine and cross-linked PNIPA hydrogels was investigated [112].

pH-sensitive microgels have been prepared by interpenetrating EPI-CD-PVA networks with poly(methacrylic acid) (PMAA) [113]. When the microgels were loaded with methyl orange, an unexpected release pattern was observed. Despite the microgels were collapsed at pH 1.4 and swollen at pH 7.4, the release rate was much faster at acid pH. This finding was due to the pH-dependent affinity of methyl orange for β-CD; the affinity being one order of magnitude larger at neutral pH when the probe was not ionized. Therefore, the pH-responsive delivery is afforded by the effect of pH on the host-guest interactions and not by the macroscopic swelling of the IPN. These results clearly highlight the key role of the CDs in controlling drug release.

The large content in hydroxyl groups makes CDs potentially useful to prepare biocompatible electrorheological fluids. These smart materials undergo rapid and reversible changes of the rheological properties under small voltage electric fields. EPI-CD networks can not endure a high electric field for a long time and the polarization is restricted owing to the rigidity and the high density in CDs [114]. Including starch during the cross-linking led to particulate networks that once mixed with silicon oil showed improved electrorheological properties [115].

It is noteworthy that some EPI-CD hydrogels have shown an enhanced ability to retain solutes owing to the numerous hydrogen bonds that CDs can form. For example, non-complexation interactions have been used to create hydrogels able to selectively recognize creatinine [116]. The hydrogels were prepared in the presence of creatinine molecules at alkaline pH. In this medium, the OH at C6s are ionized and can electrostatically interact with amine groups of creatinine. The arrangement of CDs around creatinine molecules was maintained after polymerization and removal of creatinine. Molar ratios of β-CD:creatinine 3:2 and β-CD:EPI 1:10 were found as adequate to achieve a high rebinding effect. EPI-CD networks have been also functionalized with alkyl quaternary ammonium groups to obtain traps for biliar salts. A low degree of cross-linking and the presence of ammonium groups notably enhanced the loading capability and the affinity to sodium salts of cholic, glycocholic and chenodeoxycholic acids [117].

Despite the potential of EPI-CD hydrogels, the relatively high toxicity of EPI and its pollutant character have motivated an intense search for alternative cross-linking agents to be used for pharmaceutical and biomedical applications. Diisocyanates have been extensively evaluated to prepare CD hydrogels or beads [118, 119]. Such networks have been shown particularly adequate to remove toxic substances (e.g., phenol or organic dyes) from wastewater [120]. Hydrogels of β-CD and poly(ethylene glycol) diamine, cross-linked with hexamethylene dissiocyanate, exhibited high hydrophilicity and biocompatibility as well as capability to load and to sustain the delivery of estradiol, quinine and lysozime [121]. Diisiocyanates have been also used to obtain CD-based hydrophilic hyperbranched polymers

that show a high ability to complexate guest molecules [122] and to prepare nanoporous CD particles that rapidly retain solutes from aqueous environments and release them to organic phases [123]. In this context, molecular imprinting is a quite attractive technology to improve the yield and selectivity of the loading and to achieve a better control of the release from the hydrogels [124,125]. The group of Asanuma and Komiyama have extensively explored the potential of this approach for enhancing the capability of toluene-2,4-diisocyanate cross-linked β-CD networks, in order to selectively recognize biologically relevant molecules or to remove pollutants from water streams [126]. The cross-linking in the presence of cholesterol or stigmasterol enabled the synthesis of networks in which dimers and trimers of β-CDs are gathered together to cooperately bind large steroids. After polymerization and removal of the guest molecules that served as templates, molecularly imprinted networks (MIP) are obtained. MIPs significantly took cholesterol or stigmasterol up from aqueous medium and showed much lower affinity to other structurally related steroids [127,128].

Comparative studies of the performance of β-CD hydrogels cross-linked with EPI or diisocyanates revealed the importance of the nature of cross-linker on the affinity of guest molecules for the CD cavities. For example, β-CD hydrogels and sucrose networks were prepared using EPI, succinyl chloride, hexamethylene diisiocyanate or toluene-2,4-diisocyanate in order to discriminate the role of complexation on the loading of phenol and 1-naphthol [129]. Sorption was in all cases more favourable for the CD networks than for the corresponding sucrose networks. The results suggest that diisiocyanates lead to networks of smaller mesh size and lower degree of swelling in water compared to EPI, which self-polymerizes and provides longer bridges between the CDs. Non-specific hydrophobic interactions with the network are more feasible in the case of diisiocyanate cross-linked networks, while in the EPI-CD hydrogels complexation with CDs predominates. Compared to other commercially available pollutant sorbents, CD networks showed higher sorption capacities particularly at high phenol concentrations [130]. Such a good performance together with the environmental-friendly and recyclable character make CD networks a cheap and efficient resource for cleaning water from organic pollutants and heavy metals [97,131].

An emulsion technique for the interfacial cross-linking of β-CDs with diacyl chlorides has been developed to prepare microcapsules with walls made of cross-linked CDs [132]. This capsule type-structure showed the advantage of the fast accesibility of guest molecules to the CD cavities, enabling fast loading in 5 min. Furthermore, the microcapsules were able to control the release of propranolol for several hours [133].

Condensation with poly(carboxylic acid)s have been shown as a clean way to obtain cross-linked CD networks, although it presents the drawback that the water produced during esterification requires removal at temperature above 140ºC in air or under vacuum [134]. Polyesterification of native CDs was successfully carried out with citric acid, 1,2,3,4-butanetetracarboxylic acid or poly(acrylic acid) (PAA), but failed with dicarboxylic acids. These results stress the need of using poly(carboxylic acid)s with at least three neighboring carboxylic groups separated by two or three carbons. A phosphate catalyzer (e.g., NaH_2PO_4) is also required to form the intermediate cyclic anhydride of the poly(carboxylic acid) that reacts with the CD.

The ability of CDs to react with epoxide groups under mild conditions [135] prompted the development of a method that enables the direct linking of CDs among themselves in one-step, using ethyleneglycol diglycidylether (EGDE), to obtain viscoelastic hydrogels in a fast

and predictable way [137,138]. EGDE is a relatively non-toxic reagent that posseses two epoxy groups in its structure, both of similar reactivity and able to react simultaneously, under mild conditions, with the hydroxyls groups of CDs or polysaccharides [139]. Hydroxyl ions catalyze the ring opening of the oxacyclopropane of the EGDE to react with hydroxyl groups; and at 50ºC the reaction rate is fast enough to finish the cross-linking process in a few hours without compromising the stability of the CDs and of other polysaccharides [138]. Most glycidylether groups of EGDE are consumed in the reaction and, if any still remain in the hydrogel, the washing with 0.01M HCl aq. medium opens the rings to give hydroxyl groups, resulting in highly biocompatible hydrogels [140]. For example, a minimum concentration of 10 wt-% HP-β-CD and, at least, of 14.28 wt-% EGDE were required for preparing HP-β-CD hydrogels. These concentrations enabled that 2/3 of the hydroxyl groups of each CD reacted with the cross-linker. The CD hydrogels were completely transparent and swelled in water up to a 1000 wt-% behaving as superabsorbents. Linear cellulose ethers (constituted by glycopyranose units similar to those of CDs) can be also incorpored during cross-linking to modulate the mechanical properties and the performance of the hydrogels as drug delivery systems. The content in cellulose ether (e.g., HPMC) should be in a range between the critical overlapping (0.2 wt-%) and the entanglement concentration (1 wt-%) in order to promote the homogeneous distribution of both components. Monitoring of the cross-linking process revealed that the gel time dramatically decreased as the HPMC proportion increased, mainly because the long chains of the cellulose ethers facilitate the contact between the different components. HP-β-CD hydrogels and M-β-CD hydrogels similarly prepared were tested to evaluate their ability to load and control the release of diclofenac and estradiol. The loading was made by immersion of the hydrogel in a solution of diclofenac sodium or a suspension of estradiol for one week. Both HP-β-CD and M-β-CD hydrogels were able to host twice the diclofenac concentration predicted assuming that the loading exclusively took place in the aqueous phase of the hydrogel. This behaviour proves that drug-CD complex formation indeed occurred in the CD hydrogel. Control hydrogels made of HPMC without CD released all drug in few minutes, but those made with CDs sustained the delivery for several hours. The presence of HPMC enabled to modulate the rate of the release. In the case of estradiol, particularly when autoclaving was applied during loading, the total drug uptake was up to 500 times greater than that possible in the aqueous phase. Furthermore a strong correlation between the complexation binding constant and the partition coefficient of the drug to the network was observed (Figure 8). This clearly highlights the main role of the CDs in the loading. Such a high affinity also led to sustain the delivery of estradiol for one week, and to a negative correlation between the release rate and the complexation equilibrium constant [141]. Although drug loading and release is mainly controlled by the drug/CD affinity constant, the cellulose ethers contribute to the improvement of the physical properties and to modulate the release (Figure 9).

Cross-linking with EGDE constitutes a very versatile method to prepare hydrogels of CDs incorporating other structurally related polymers, such as dextran. A comparative study of the performance of HP-β-CD (20%) hydrogels prepared in presence of 0.4 or 0.8% HPMC, methylcellulose (MC), hydroxypropyl cellulose (HPC), carboxymethyl cellulose (CMCNa), or dextran, as carriers of sertaconazole was recently carried out [142]. Sertaconazole is an antifungal agent very effective for treatment of *Candida albicans* infections; however its poor aqueous solubility is still a challenging issue for developing suitable formulations. HP-β-CD-

based hydrogels provided a microenvironment very rich in CD cavities responsible for hosting the drug and controlling its release rate. All the hydrogels were superabsorbents, though those containing MC, CMCNa or HPC were the ones with the lowest degree of swelling. This phenomenon can be attributed to the concomitance of two effects: i) a less hydrophilic character compared to HP-β-CD and ii) a higher degree of cross-linking due to an easier reaction of EGDE with the unsubstituted hydroxyl groups of cellulose. The hardness and compressibility of hydrogels prepared with HPMC or dextran were similar to those of the HP-β-CD sole hydrogel (4.2 N and 3.1 N·mm). The addition of other polyssacharides caused, in general, an increase in these parameters (up to 9.4 N and 8.7 N·mm), which confirms the greater apparent cross-linking density of HP-β-CD/MC, HP-β-CD/CMCNa and HP-β-CD/HPC hydrogels. Sertaconazole loading was carried out by immersion of hydrogels in drug suspensions. Application of autoclaving during hydrogel loading revealed that this sterilization treatment still promoted the drug uptake by the CDs and did not cause relevant changes in the mechanical properties of the hydrogels. Non-treated HP-β-CD hydrogels loaded 21.7 mg/g and, when autoclaved, 18.7 mg/g. Hydrogels containing MC or HPMC loaded similar amounts or even greater. By contrast, non-autoclaved hydrogels made with HPC, CMCNa or dextran showed a significantly lower loading capability. Once autoclaved, HP-β-CD/HPC hydrogels reached similar values to those obtained with HP-β-CD sole hydrogels. In the case of CMCNa or dextran hydrogels, autoclaving enhanced the loading to such a high extent that these hydrogels became the ones with the greatest loading capability. All hydrogels showed a relatively fast delivery of drug in the first 24 hours, followed by a more sustained release step up to 4 days. The antifungal effectiveness of the sertaconazole-loaded hydrogels was confirmed using *Candida albicans* cultures in exponential phase of growth. Therefore, the EGDE cross-linked CD-polysaccharide hydrogels have a great potential as efficient carriers of antifungal drugs to be applied topically or on mucosa.

Following a similar approach, HP-β-CD hydrogels with domains of interpenetrating poly(acrylic acid) (PAA) micro-networks (Carbopol®) were developed with the aim of combining the pH-responsiveness and mucoadhesive properties of carbopol and the performance of cross-linked CDs [143]. The threading of the growing chains of cross-linked CDs through the micronetworks of carbopol immobilizes the microgels preventing their leaking from the system, without needing further cross-linking of the acrylic component. The resulting structure in which the CD network is continuous but the carbopol network is discontinuous, presents microdomains of IPN (micro-scale IPN). The following benefits of the micro-scale IPNs are pursued: i) no previous preparation of acrylic monomers of CD is required; ii) no lost of components by leaking as may occur for semi-IPNs of linear PAA; iii) pH-responsiveness and bioadhesion are expected due to the cross-linked PAA (Figure 10); iv) the discontinuous structure may facilitate the relative movement of both networks and thus excellent mechanical properties are expected; and v) the composite is obtained in a one-step procedure.

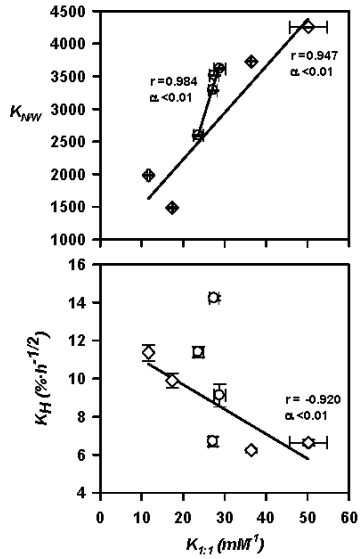

Figure 8.- Dependence of network/water partition coefficient (K_{NW}) and of the release rate constant (K_H) of estradiol for HP-β-CD (◊) and M-β-CD -based hydrogels (○) on the estradiol:cyclodextrin stability constants ($K_{1:1}$). Reproduced from [141] with permission from Elsevier.

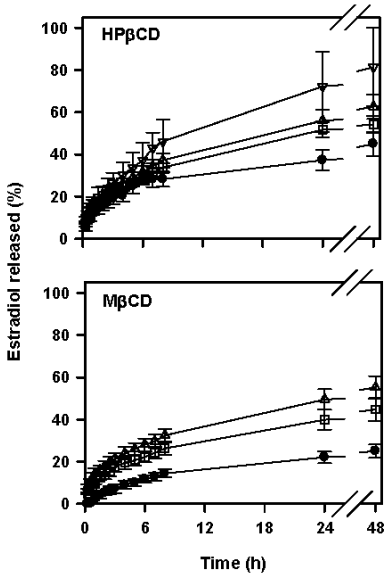

Figure 9- Estradiol release profiles from HP-β-CD or M-β-CD hydrogels prepared with 20% cyclodextrin (●), 20% cyclodextrin – 0.25% HPMC (□), 25% cyclodextrin – 0.25% HPMC (Δ), and 30% cyclodextrin – 0.25% HPMC (▽), which were previously loaded in aqueous suspensions of estradiol. Reproduced from [141] with permission from Elsevier.

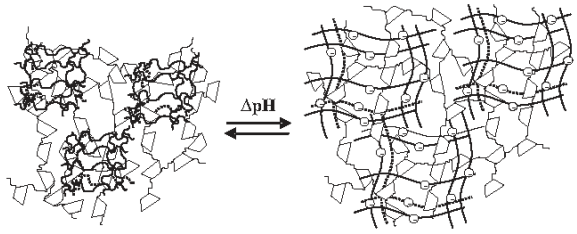

Figure 10. Schematic draw of the pH-sensitive swelling of HP-β-CD/ carbopol micro-scale IPNs. Reproduced from [143] with permission from Elsevier.

The micro-scale IPNs were evaluated as platforms for estradiol and ketoconazole release [143]. An increase in carbopol content from 0.2 to 1 wt-% caused a decrease of the hardness (from 3.0 to 0.7 N), the compressibility (from 2.5 to 0.5 N) and the modulus of deformability (from 14 to 2.1 kPa); but an increase in the bioadhesion force (from 0.14 to 0.70 N·mm). The micro-scale IPNs loaded much more drug (up to 200-fold) than that dissolved in the aqueous phase. The drug loading was enhanced even by carbopol. This behavior stems from the fact that when the CD is cross-linked in the presence of the microgels, the network structure is more open and can swell more and then the drug can easily find the CD to form the complexes. All these micro-scale IPNs sustained the release for several days; the rate being also dependent on carbopol content and pH (Figure 11). Therefore, an adequate design of the HP-β-CD/carbopol micro-scale IPNs provides a single material with tunable mechanical properties, in which the complexation ability of CDs is combined with the bioadhesive and pH-responsive features of PAA.

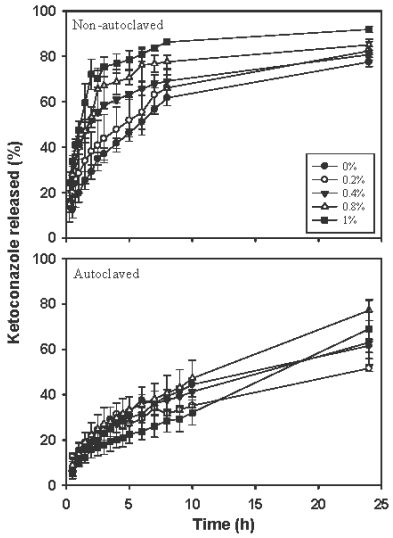

Figure 11. Ketoconazole release profiles in 0.3% SDS solution (pH 7.8) from HP-β-CD/ carbopol micro-scale IPNs that were prepared with different proportions in carbopol (see legend) and that were previously loaded by immersion in a drug suspension applying autoclaving or not. Reproduced from [143] with permission from Elsevier.

Hydrogels Obtained by Copolymerization of CD Monomers

Acrylic hydrogels have a long tradition as components of biomedical devices owing to the versatility of their mechanical properties and to their hydrophilic character that make the diffusion of small nutrients and oxygen possible. Nevertheless, such a hydrophilicity prevents the effective loading of hydrophobic drugs in the aqueous environment of the network and, on the other hand, promotes the rapid release of hydrophilic drugs. Incorporation of CDs to the acrylic hydrogels can overcome both limitations if the CDs can form complexes with the target drug. This approach requires the previous formation of a monomer of CD able to copolymerize with other monomers used to form the hydrogel.

Several synthetic routes of CD monomers have been developed. Most chemical modifications of CDs take benefit of the hydroxyl groups of the glucopiranose ring. Considering that CDs contain 18 (α-CD), 21 (β-CD) or 24 (γ-CD) substitutable hydroxyl groups, the number of possible derivatives is unlimited. The total number of hydroxyl groups is kept in the case of the hydroxyalkylated derivatives, but decreases when the substituent has a different nature. The hydroxyl groups at C6 are the most alkaline ones and, thus, the most nucleophile. The hydroxyl groups at C2 are more acid while those at C3 are quite inaccessible [9]. The presence of a high number of equally reactive hydroxyl groups makes the preparation of monofunctionalized monomers particularly challenging. Thus, in most publications, multifunctional monomers are reported.

First attempts to prepare monofunctional monomers of CDs involved the reaction of α-CD and β-CD with m-nitrophenyl ester in alkaline medium, at room temperature for 5 min. The nitrophenyl esters form complexes with the CDs and lead to selective transesterification at one of the secondary hydroxyl groups, minimizing multifunctional products and suppressing thermal polymerization [144]. Acryloyl and N-acrylyl-6-aminocaproyl monomers of CD were polymerized among themselves or with other water soluble monomers to render water soluble polymers [144]. Compared to natural β-CD, the polyacryloyl-β-CDs showed a lower affinity constant for small molecules such as m-chlorobenzoic acid and cinnamic acid, but higher for large substrates with two aromatic rings such as methyl red and orange I. These results suggest that there is a cooperative action of the β-CD units on the polymer chain in the binding of large substrates [145]. Copolymerization of acryloyl-β-CDs with N-isopropylacrylamide (NIPA) has been shown useful to prepare porous hydrogels that undergo volume phase transitions very rapidly when immersed in aqueous medium at 37ºC [146]. The group of Asanuma and Komiyama has intensively explored the development of molecularly imprinted networks for certain molecules able to simultaneously complexate with several CD units. Acryolyl-α-CD and acryloyl-(6-O-α-D-glucosyl)-β-CD were cross-linked in the presence of various guest molecules (vancomycin, cefazolin, phenethicillin, and some dipeptides) to obtain rigid particles with a microstructure able to fit the complexation preferences of each guest molecule [147]. In average, the imprinted networks were able to load twice the amount of drug loaded by the CD networks prepared in the absence of the guest molecule. Piletsky *et al.* found that when the hydrophobic selective binding capacity of bisacryloyl-β-CD monomers is combined with functional monomers able to interact electrostatically (2-acryloylamido-2,2′-dimethylpropane sulfonic acid), networks with an enhanced affinity for amphiphilic molecules, such as phenylalanine, can be obtained. The networks can even distinguish the enantiomers of phenylalanine [148,149].

Monotosyl derivatives of β-CD can be obtained by reaction between one C6 primary hydroxyl group of β-CD and tosyl chloride [150]. The 6-OTs-β-CD precursor was used to functionalize polyvinylamine for chromatographic purposes [151], to improve the performance of natural polymers as drug carriers [152], and to prepare new monofunctionalized monomers [153]. For this last goal, ethylenediamine (EDA) or 1,6-hexanediamine (HAD) reacted with mono-6-OTs-β-CD to obtain β-CDs containing one primary amino group. Then, the amino group reacted with glycidyl methacrylate (GMA) to render monomethacrylate β-CD monomers (GMA-EDA-β-CD and GMA-HDA-β-CD; Figure 12).

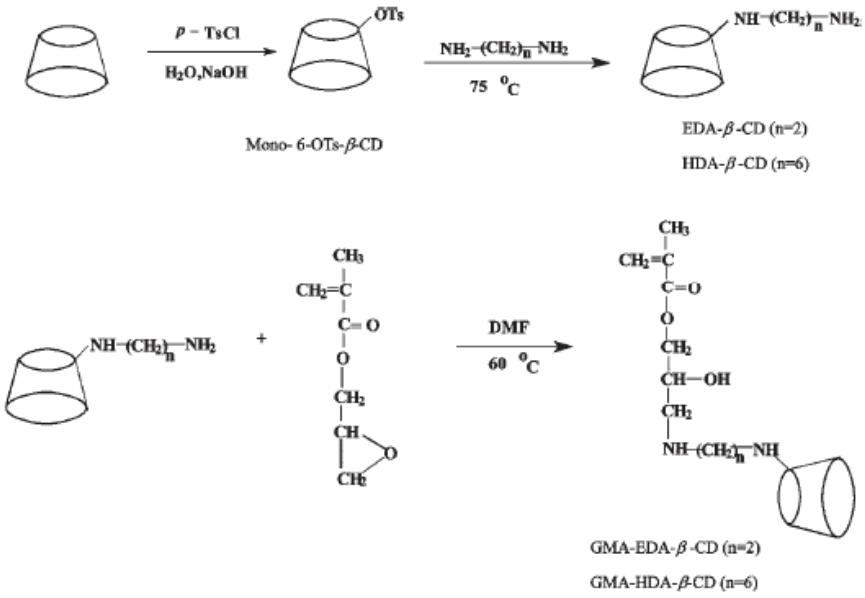

Figure 12. Route of synthesis of monomethacrylate β-CD monomers (GMA-EDA-β-CD and GMA-HDA-β-CD). β-CDs with primary amino groups are obtained by reaction of mono-6-OTs-β-CD with ethylenediamine (EDA) or 1,6-hexanediamine (HAD) and then they react with glycidyl methacrylate (GMA). Reproduced from [153] with permission of Wiley-VCH Verlag GmbH &Co.

Copolymers of N-isopropylacrylamide (NIPA) and these monomethacrylated β-CD derivatives (GMA-EDA-β-CD and GMA-HDA-β-CD) showed high water solubility while maintaining the temperature responsiveness of PNIPA. Attemps to prepare linear copolymers by polymerizing first NIPA with GMA and then coupling with the ethylenediamine derivative of mono-6-Ts-β-CD failed due to the very slow conjugation rate and the rapid cross-linking among NIPA-GMA chains that took place during drying [154]. Nevertheless, the resultant hydrogels were able to change the degree of swelling as a function of temperature and to uptake methyl orange with an affinity similar to that exhibited by the ethylenediamine derivative of mono-6-Ts-β-CD sole. Further experiments involved the preparation of hydrogels by free radical copolymerization of aqueous solutions of 2-hydroxyethyl acrylate (HEA; 87-100 mol%) with GMA-EDA-β-CD (0-13 mol%). HEA homopolymerization

occured faster than the copolymerization with GMA-EDA-β-CD and, therefore, the actual content in CD in the hydrogels was again much lower than expected. In general, the presence of GMA-EDA-β-CD led to a much higher glass transition temperature (enhanced network rigidity) and a lower degree of swelling, but also to a slower release of melatonin (from 90% to 70% release at 120 min) [155].

Acrylamidomethyl-CD can be easily prepared from the acid-catalyzed reaction of N-methylolacrylamide (NMA) and CD [156]. This procedure is carried out in aqueous environment, which is very convenient to prepare materials intended for biomedical applications. NMA-β-CD with acrylamidomethyl group contents ranging from 1 to 3 per CD molecule was used to functionalize cotton fibers [156]. Based on the same procedure, monosubstituted NMA-γ-CD was synthesized using a NMA:γ-CD mol ratio of 10; the reaction being complete in less than 30 min. The copolymerization of NMA-γ-CD and sodium acrylate (3-4 M) generated pH-responsive hydrogels able to load and control the release of two drugs with recognized ability to form inclusion complexes: triamcinolone acetonide (TA) which is almost insoluble in water and did not interact with carboxylic acid groups, and propranolol (PR) a fairly soluble drug that electrostatically interacts with acrylic networks.

NMA-γ-CD monomer is highly soluble in water and thus the free radical polymerization was carried out in aqueous solution in the presence of BIS (13-39 mM) as cross-linker [157]. Regardless the NMA-γ-CD ratio, swollen hydrogels were highly flexible and transparent. The glass transition temperature of the acrylate network (130ºC) was not modified when copolymerised with NMA-γ-CD, which means that the CD monomer did not act as plasticizer and was effectively attached to the network. Hydrogels prepared without NMA-γ-CD loaded minimal amounts of TA, owing to the lack of the affinity of the drug for the hydrogel components and to the low degree of swelling in the ethanol/water medium used for TA loading. When the amount of NMA-γ-CD used in the synthesis was 15 mg/ml or higher, the hydrogels could notably swell and sorb the drug. NMA-γ-CD hydrogels were able to sustain the release of TA for 24 h disregarding the pH of the medium, indicating that the drug:CD affinity governs the delivery process. In the case of PR, the highest loading was observed for acrylate hydrogels without NMA-γ-CD, which was related to a diminishing in the content in acrylic acid groups per mass of hydrogel as the content in NMA-γ-CD increases. As in the case of TA, PR release was not controlled by the pH-dependent swelling of the hydrogels either. The release was slower at pH 7.4 when the hydrogels were fully swollen, confirming the role of drug-network interactions in the control of the release.

In this context, it is worth stressing that the position of the acrylamide group in the glucopyranose ring may strongly determine the functionality of the network. For example, acrylamide monomers of β-CD that tether the reactive double bond on either the wider or the smaller rim of the truncated cone were synthesized for preparing networks able to recognize amino acid derivatives and oligopeptides in water [158]. The target molecules were added to the monomers soup in order to induce the arrangement of the CD monomers in such a way that after polymerization and removal of the template molecules, the spatial distribution of the monomers is maintained, leading to favorable binding sites for selective recognition and hosting of the target molecules. Since molecules able to be hosted in the CD cavity take a specific orientation, the position of the reactive double bond on the CD monomer enormously affects the imprinting. The reactive double bond determines the distance between the template

and the polymerizable moiety. Most molecules that form complexes with β-CD enter through the larger rim [159]. This is also observed for protected amino acids (e.g. N-benzyloxycarbonyltyrosine in Figure 13) irrespectively of the position of the vinyl group. Therefore, the preorganized monomers of β-CD, mono-3-(N-acrylamido)-3-deoxy-altro-β-CD (3-AAm-CD) and mono-6-(N-acrylamido)-6-deoxy-β-CD (6-AAm-CD) rendered after polymerization two networks of quite different microstructure (Figure 13). 3-AAm-CD provided receptor cavities for N-benzyloxycarbonyltyrosine much smaller and adapted to the molecular shape of this amino acid. By contrast, the copolymerization occured far from the template when 6-AAm-CD, which has the vinyl group protruding towards the opposite site of the template, was used. This resulted in wider cavities, much larger than the template. As a consequence, 3-AAm-CD networks showed highly precise recognition of the template molecule, i.e. N-benzyloxycarbonyltyrosine, and exhibited a low capability to host larger amino acids and peptides. The opposite was true for 6-AAm-CD [158]. Recently, 6-AAm-CD has been found useful to create networks with artificial receptors that clearly distinguished angiotensin I and angiotensin II, despite the apparent similarity of amino acid sequences of these two oligopeptides. The excellent results obtained when used as the stationary phase of HPLC indicate that the molecular imprinting is not directly associated with the primary structure of the oligopeptide template, but with the conformation of the oligopeptide in solution [160].

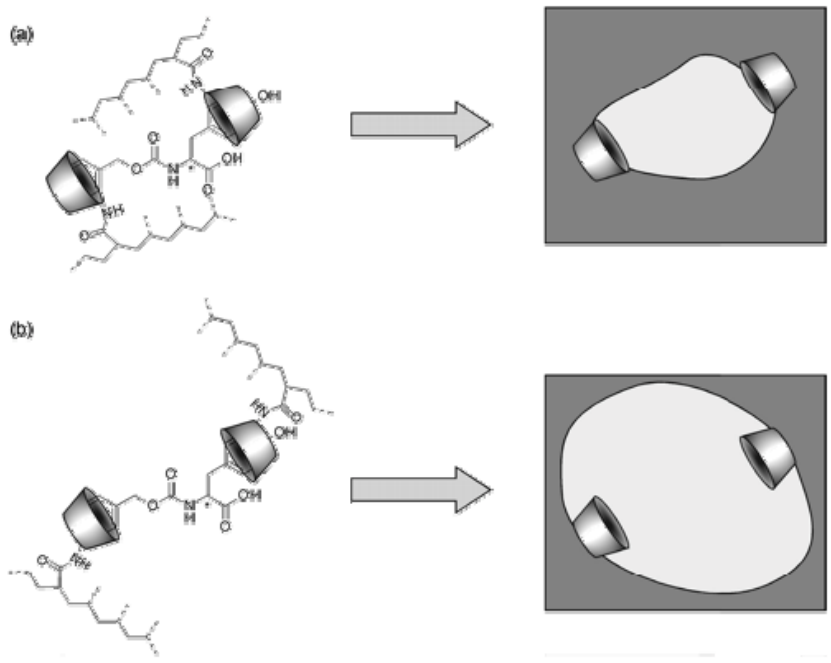

Figure 13. Arrangement of mono-3-(N-acrylamido)-3-deoxy-altro-β-CD (a) or mono-6-(N-acrylamido)-6-deoxy-β-CD (b) during polymerization and cross-linking with N,N′-methylenebis(acrylamide) in the presence of N-benzyloxycarbonyltyrosine. Reproduced from [158] with permission of the American Chemical Society.

Condensation of CDs with maleic anhydride (MAH) has been proposed to obtain smart monomers that combine the ability of CDs to host guest molecules and the pH-sensitiveness of carboxylic groups. The number of vinyl and carboxylic acid groups per CD can be effectively controlled by tuning the MAH:β-CD mole ratio [161]. PNIPA(91-64 %w/w)-co-MAH-β-CD(9-36% w/w) hydrogels, which were prepared by free radical polymerization of NIPA and MAH-β-CD in aqueous solutions at 20ºC, showed temperature-, pH-, and ionic strength-responsiveness. Interestingly, the changes in the degree of swelling induced by these variables were reversible and reproducible after several cycles, exhibiting a true intelligent behaviour [161]. PNIPA-co-MAH-β-CD hydrogels have been proposed to uptake chlorambucil and to release it at pH-dependent rate [162]. Hydrogels sensitive to both pH and ionic strength have been also obtained by electron beam irradiation of MAH-β-CD: acrylic acid (AA) aqueous solutions at 1:3, 1:3.5, 1:4, 1:4.5 and 1:5 w/w [163]. The hydrogels were collapsed at pH ranging from 1 to 3 and showed an abrupt swelling from pH 3 to 7, particularly when the ionic strength of the medium was low.

MAH-β-CD has been also copolymerized with macromonomers of Pluronic® F68 and poly(ε-caprolactone). As mentioned in previous sections, Pluronic represents a family of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) block copolymers that undergo temperature-sensitive sol-gel transitions [164]. *In situ* gelling may occur when Pluronic solutions enter into contact with the body fluids, which makes Pluronic particularly attractive for preparing drug depots and for tissue engineering. Nevertheless, the gels usually exhibit poor mechanical properties. Cross-linking of reactive PEO-PPO-PEO diacrylate derivatives generate mechanically stable hydrogels [165]. Introduction of poly(ε-caprolactone) segments prior to the incorporation of vinyl groups renders the networks biodegradable [166]. UV-induced copolymerization of acryloyl monomers of Pluronic F68-g-poly(ε-caprolactone) (5-30%) with MAH-β-CD (0-25%) was carried out in water (70%). Since the MAH-β-CD monomer possesses several vinyl groups, it could act as cross-linker of the hydrogel. The greater the content of MAH-β-CD is, the higher the storage (G′) and loss (G′′) moduli. Therefore, changing the MAH-β-CD content enables the fine tuning of the mechanical behaviour of the hydrogels [167]. The capability of such hydrogels to uptake drugs and to control their release has not been tested yet. A recent attempt to create biodegradable CD networks involved the copolymerization of poly(D,L-lactic acid) (PLA) macromonomer with a β-CD derivative (both obtained by reaction with 1-allyloxy-2,3-epoxy propane). The microgels were prepared by free radical polymerization in dimethyl sulfoxide/toluene medium at 70ºC. The content in β-CD monomer and the number of reactive double bonds enabled the regulation of the hydrolysis rate of the microgels in phosphate buffer at 37ºC [168].

A multifunctional β-CD urethane-methacrylate monomer has been recently synthesized according to a two-step addition mechanism. First, an urethane-methacrylate derivative was synthesized adding hydroxyethylmethacrylate (HEMA) to toluene-2,4-diisocyanate, and then such a derivative reacted with β-CD to obtain the β-CD monomer [169]. Hydrogels were prepared by UV-irradiation of HEMA (87.5-90 mol%), urethane-methacrylate-β-CD monomer (0-2.5 mol%) and cross-linker poly(ethylene glycol) diacrylate. The presence of the β-CD monomer at 2.5 mol% caused a remarkable increase in the degree of swelling of the hydrogel (from 34 to 50%) and on the amount of salicylic acid, sulfathiazole, rifampicine and

methyl orange that the hydrogels were able to take up. Nevertheless, the effect on drug release was not homogeneous, slightly delaying the delivery of the hydrophilic methyl orange and salicylic acid, but accelerating the release of the hydrophobic sulfathiazole (Figure 14). Such a behavior may be related to the different affinity constants of the drugs for the CDs and to the different solubility of the drugs in the buffer used as the release medium. Both variables should be taken into account to interpret the release from CD-hydrogels.

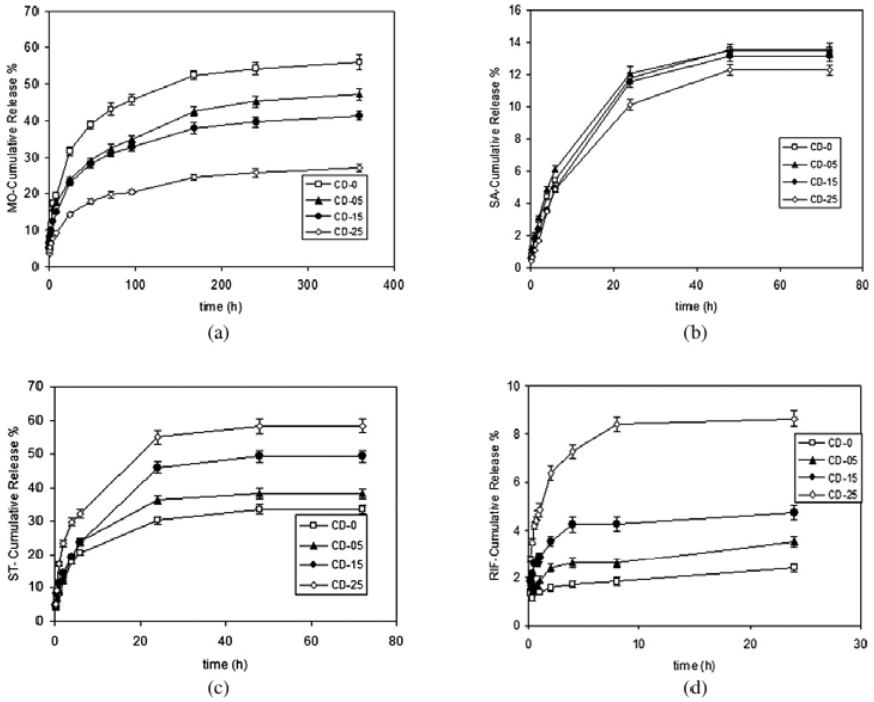

Figure 14. Cumulative drug release from β-CD-UM based hydrogels: (a) methyl orange, (b) salicylic acid, (c) sulfathizaole, and (d) rifampicin. Reproduced from [169] with permission of John Wiley & Sons, Inc.

Methacrylic monomers of CDs have attracted a great interest for a wide range of purposes and even the monomer 2-hydroxy-3-methacryloyloxy-propyl-β-CD (βW7MAHP from Wacker-Chemie GmbH) with an average number of 2.5 double bonds per HP-β-CD unit was commercially available for some time. This monomer can be prepared by reaction of glycidyl methacrylate (GMA) with HP-β-CD in alkaline medium [170]. Cross-linked βW7MAHP and copolymers of βW7MAHP with 2-hydroxyethylmethacrylate (HEMA) have been shown useful for the removal of pollutants from water [171]. In particular, copolymerization with HEMA notably enhanced the degree of swelling in water and, thus, the sorption capacity of the βW7MAHP networks due to a better accesibility of the CD cavities. Copolymerization of βW7MAHP with 1-vinyl-2-pyrrolidinone rendered soluble polymers when the content in βW7MAHP was below 60 mol%, while greater proportions led to cross-linked hydrogels [170].

Methacrylic monomers of CDs with 1 to 6 double bonds per CD unit can be also obtained via a single step reaction of β-CD with methacrylic anhydride using sodium hydroxide as catalyst [172]. Direct photopolymerization of 6% methacrylic-β-CD solutions enabled the formation of hydrogels, while concentrations above 8% produced brittle and white networks. CD monomers having methacrylic groups only at positions 2 and 3 have been obtained by acetylation of primary hydroxyl groups and esterification of secondary hydroxyl groups with methacrylic anhydride [173]. Such CD monomers have been successfully used as templates during polymerization of other methacrylate monomers in order to achieve degrees of polymerization ranging between 10 and 14 [174]. The complete esterification of primary (7) and secondary (14) hydroxyl groups enabled the preparation of (2,3-di-O-methacrylated-6-methacrylated)-β-CD monomer which served as template of methacrylic acid oligomers with degrees of polymerization 7 and 14 [175] (Figure 15). Complexation of methacrylic-β-CD monomer with guest molecules notably modifies the spatial arrangement of the reactive double bonds and the performance of the monomer for template polymerization [176].

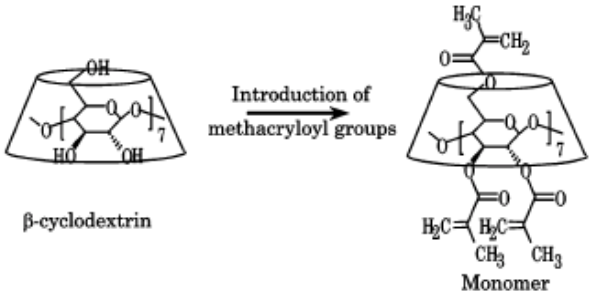

Figure 15. The complete esterification of primary and secondary hydroxyl groups enables the preparation of (2,3-di-O-methacrylated-6-methacrylated)-β-CD monomer. Reproduced from [175] with permission of the American Chemical Society.

Methacrylated monomers have been also explored in detail for preparing dental fillings. Incorporation of common photoinitiators, such as camphorquinone and ethyl-4-dimethylaminobenzoate, results in the formation of inclusion complexes with the monomers, which significantly alters the degree of conversion and the flexural strenght of the fillings [177,178]. Optimization of the ratio of polymerizable methacrylate groups/hydroxyl groups enabled the preparation of adhesive CD monomers that promote the bonding of dental composites to dentin [179]. Resins prepared with 33% methacrylated-β-CD, 30% HEMA, and 37% acetone showed the maximum shear bond strenght (16 MPa), with values similar to those exhibited by commercial products.

Taking into account this previous information about methacrylate-β-CD monomers, loosely cross-linked hydrogels made of HEMA and (2,3-di-O-methacrylated-6-methacrylated)-β-CD were designed with the aim of obtaining foldaway and viscoelastic networks useful for the development of medicated soft contact lenses (SCL). The limited ocular bioavailability achieved with conventional ophthalmic formulations has prompted the search of alternatives, among which SCLs able to combine the capability to correct optic

defficiencies with the capability of loading a drug and to control its release are one of the most promising candidates [180]. Nevertheless, most SCLs cannot uptake therapeutic doses of hydrophobic drugs neither control the delivery of the hydrophilic ones. Differently from β-CD which is not soluble in the liquid HEMA, (2,3-di-O-methacrylated-6-methacrylated)-β-CD enabled the synthesis of hydrogels without using solvents. The presence of CDs in the network (0.23-1.82 mol%) led to transparent hydrogels that showed a high cytocompatibility and did not induce macrophage response [181]. The greater the content in methacrylated β-CD was, the higher the glass transition temperature, the lower the degree of swelling and free water proportion, and the greater the storage and loss moduli of the swollen hydrogels. These findings were directly related to the increase in cross-linking degree caused by the methacrylated β-CD. 3-Methyl benzoic acid (3-MBA) that has a high affinity for β-CD ($1.3 \cdot 10^7$ M^{-1}) was used as a probe to gain an insight into the accessibility to β-CD cavities once attached to the pHEMA network, and their capability to form complexes with small drug molecules. Control pHEMA hydrogels loaded a small amount of 3-MBA mainly in the aqueous phase (Figure 17). pHEMA-co-βCD hydrogels were able to uptake greater amounts of 3-MBA, although a progressive decrease in the drug:βCD molar ratio was observed as the proportion of β-CD monomer increased. Hydrogels with a low content in β-CD loaded more than one 3-MBA molecule per β-CD, owing to formation of 3-MBA dimmers. Oppositely, in hydrogels prepared with a high β-CD monomer proportion (> 0.167 g/ml) the complexation capability of the CDs was not fulfilled, which can be attributed to a smaller mesh size (i.e. greater cross-linking degree and lower content in water) that hinders the diffusion of 3-MBA. Additionally, the contribution of steric impediments owing to the proximity of the β-CD units in the network cannot be discarded. Nevertheless, even in those hydrogels prepared with the greatest β-CD proportion, 42% of the cavities participate in complexation (Figure 16).

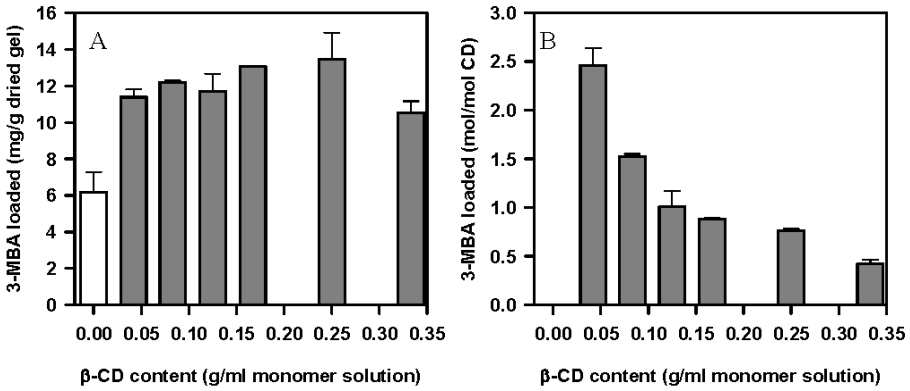

Figure 16. Total (A) and relative (B) amounts of 3-MBA loaded by hydroxyethyl methacrylate-co-2,3-di-O-methacrylated-6-methacrylated-βCD hydrogels. Data from [181].

Drug-loading studies were carried out with hydrocortisone and acetazolamide, both of practical interest for the local treatment of ocular pathologies and able to form complexes with CDs, in water and in lachrymal fluid. Hydrocortisone loading progressively decreased as the content in methacrylated-βCD rose due to a decrease in the volume of aqueous phase of the hydrogel. Acetazolamide loading showed a maximum for an intermediate content in β–CD (0.125-0.167 g/ml) owing to a balance between complexation with β-CD and hydrogel mesh size (Figure 17). In fact, these hydrogels showed a 2-fold (3-fold when autoclaved) increase in acetazolamide loading compared to the hydrogels prepared without β-CD. The hydrogels sustained hydrocortisone delivery for 7 days. The acetazolamide release rate was dependent on the β-CD content and could be prolonged for 24 days (Figure 18). In sum, an adequate selection of the content in β-CD enables to obtain pHEMA-co-βCD hydrogels suitable for specific biomedical applications.

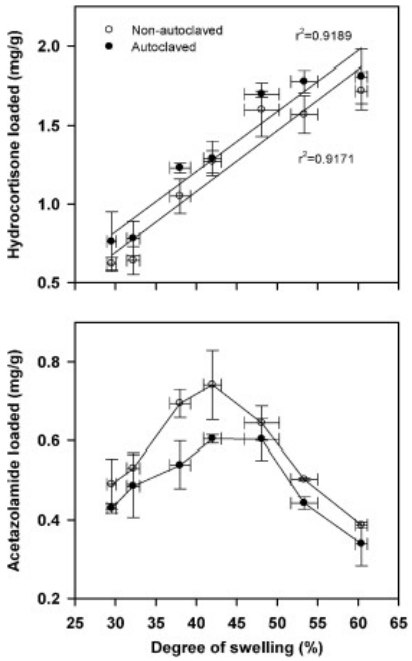

Figure 17. Dependence of the amount of drug loaded on the degree of swelling of pHEMA hydrogels prepared with 80 mM EGDMA and different amounts of methacrylated-β-CD. Reprinted from [181] with permission from Elsevier.

CD Functionalization of Preformed Networks

Functionalization with CDs of preformed materials is been explored for a broad range of purposes. In general, materials have to combine appropriate bulk and surface properties for being suitable for a certain application. The bulk properties, such as strenght, toughness and chemical and mechanical stability, influence the long-term durability of the material, while

the surface properties govern the interfacial interactions and performance when enters into contact with foreing compounds or surfaces (e.g., other materials or the living tissues). Surface functionalization with CDs opens the possibility of modulating the affinity of the surface (particularly when highly hydrophilic) towards certain molecules. For example, textil materials are coupled with CDs with the aim of retain colours, fragances, insect repellents or even antimicrobial substances [182-184]. Cotton with immobilized CDs shows a slower volatilization of fragances and can even stand up to 15 washes retaining the fragance [185]. In the biomedical field, surface modification of polymeric medical devices with CDs resulted in a lower adsorption of proteins and enhanced blood compatibility [186].

In the particular case of hydrogels, functionalization with CDs was motivated by the aim of maintaining the bulk properties of networks that had been shown adequate for specific purposes. As mentioned in previous sections, CD monomers usually have more than one reactive double bond and, therefore, they act as cross-linking points altering the viscoelastic, mechanical and swelling characteristics of the hydrogels. Thus, attachment of CDs to preformed hydrogels is envisioned as a way to keep the favourable bulk properties of the networks and to provide them with new functionalities. Two recent examples of this successful approach are described below.

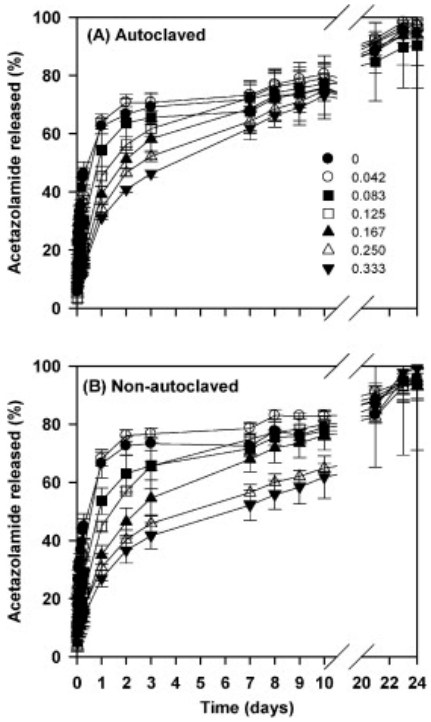

Figure 18. Acetazolamide release profiles from pHEMA hydrogels prepared with 80 mM EGDMA and different amounts of methacrylated-β-CD: 0 (●), 0.042 (■), 0.083 (□), 0.125 (○), 0.167 (▲), 0.250 (△), 0.333 (▼) g ml^{-1} of monomeric solution. Reprinted from [181] with permission from Elsevier.

Hydrogels able of undergo autonomous shrinking/swelling phase transitions accompanied by the complexation/decomplexation of a guest molecule were prepared using β-CD as the sensing moiety and NIPA as the actuating moiety [187]. NIPA (20 g) was copolymerized with p-nitrophenyl acrylate (3.4 g) in N,N′-dimethylformamide. Then, aminated CDs were incorporated to the p-nitrophenyl acrylate moities using an ester exchange reaction. CD complexation of a guest molecule changed the hydrophilic/hydrophobic balance of the network and, consequently, the transition temperature. Particularly, complexation of 8-anilino-1-naphthalene-sulfonic acid creates a hydrophobic microenvironment that decreases the transition temperature, while decomplexation reestablishes the transition temperature. At the same time, the phase transition alters the complexation; shrinking makes the complex unstable and leads to decomplexation. Coordinating these two mutual effects at a temperature in between that of the hydrogel transition when CDs are forming complexes and that observed when CDs are free, an autonomous oscillatory phenomenon was achieved (Figure 19). These hydrogels have potential as components of sensors.

Attachment of CDs to acrylic hydrogels enabled the development of SCL that maintain the mechanical properties, the swelling degree, the oxygen permeability and the biocompatibility of the starting hydrogels, but show a notably improvement of their ability to load drugs and to control their release rate [188]. Poly(hydroxyethylmethacrylate) hydrogels were prepared by copolymerization with glycidyl methacrylate (GMA) at various proportions and then β-CD was grafted to the network by reaction with the glycidyl groups under mild conditions. This led to networks where the β-CDs form no part of the structural chains but they are hanging on 2–3 ether bonds through the hydroxyl groups (Figure 20). The pendant β-CDs enhanced diclofenac loading by 1300% and drug affinity 15-fold and provided the hydrogels with the capability of sustaining drug delivery in lacrimal fluid for two weeks [189].

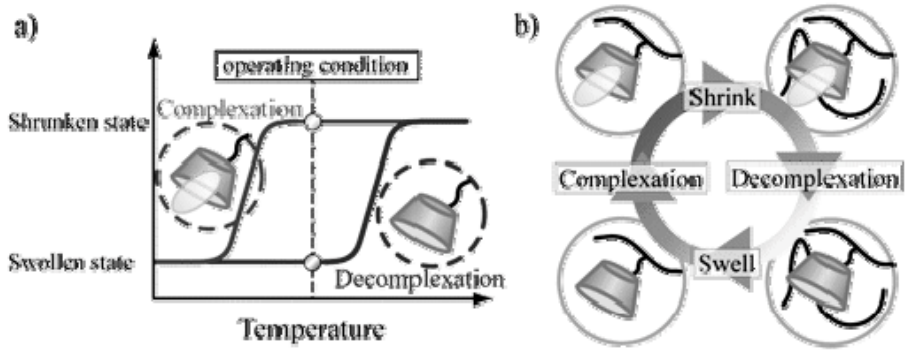

Figure 19. (a) Operating temperature for autonomous oscillatory phenomenon, which would be realized in nonequilibrium open system of poly(NIPAM-co-CD)/ 8-anilino-1-naphthalene-sulfonic acid, and (b) schematic description of the autonomous oscillatory phenomenon in the open system: polymer shrinking (upper process), decomplexation of 8-anilino-1-naphthalene-sulfonic acid (right-hand process), polymer swelling (lower process), and complexation of 8-anilino-1-naphthalene-sulfonic acid (left-hand process). Reproduced from [187] with permission of the American Chemical Society.

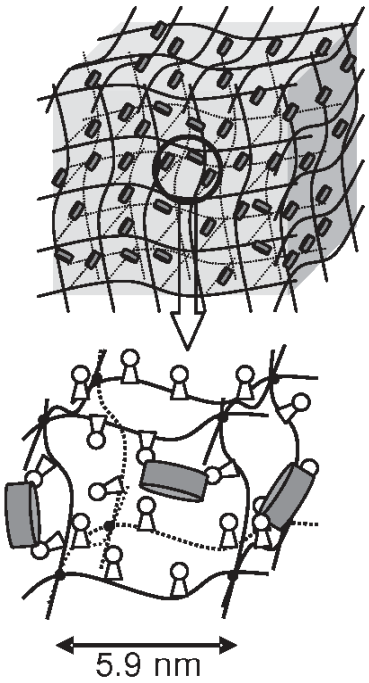

Figure 20. Scheme of a pHEMA-co-GMA hydrogel with pendant βCDs. Reproduced from [189] with permission of Elsevier.

CONCLUSION

The active research in the field of CD-based hydrogels has opened new perspectives for preparing advanced pharmaceutical and biomedical devices. Diverse strategies for the design of biocompatible materials covering a wide range of mechanical and drug loading/release features are under fast development. Therefore, one can envision that in years to come techniques to create tailor-made CD-materials capable of facing specific demands will be available.

ACKNOWLEDGEMENTS

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Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto.

P ES 200802364; (PCT/ES 2009/070333).

Número de prioridade: Data de prioridade:

Número ITP: 61851 (recibido el 26 de xuño de 2008)

Título:

Hidrogeles acrílicos con ciclodextrinas, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto.

Inventores:

ALVAREZ LORENZO, Carmen; ROSA DOS SANTOS, José Fernando; TORRES LABANDEIRA, Juan José; CONCHEIRO NINE, Angel.

Carmen Alvarez Lorenzo: ffrusdog@usc.es

Departamento de Farmacia y Tecnología Farmacéutica

Facultad de Farmacia

Universidade de Santiago de Compostela

15782- Santiago de Compostela (España)

Telf. 981563100, Ext. 14876, 14877

Resumen :

Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto. Procedimiento de obtención de hidrogeles acrílicos con ciclodextrinas colgantes caracterizado porque los hidrogeles están constituidos por un entramado polimérico obtenido por polimerización de monómeros acrílicos o metacrílicos mono- y bifuncionales y monómeros que cuentan con grupos glicidilo en su estructura, al que una vez formado se unen covalentemente unidades de ciclodextrina; y el uso y aplicaciones de las composiciones en la preparación de lentes de contacto con capacidad para incorporar fármacos, sustancias activas o demulcentes útiles en el tratamiento de estados patológicos o fisiológicos, en la elaboración de sistemas de liberación tópica, transdérmica o transmucosal de medicamentos o sustancias activas y en la preparación de cosméticos.

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UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
EDIFICIO CACTUS- CAMPUS SUR
15782 SANTIAGO DE COMPOSTELA
A CORUÑA

Madrid, 07 de agosto de 2008

Asunto: ENVIO DOCUMENTOS

Remito a Vd. los siguientes documentos correspondientes a **su solicitud de patente nº P200802364.**

X- Copia de la Solicitud
- Justificante de pago

OFICINA ESPAÑOLA DE PATENTES Y MARCAS (S.G.)
Salida
Nº. 200800015648
08/08/2008 12:03:40

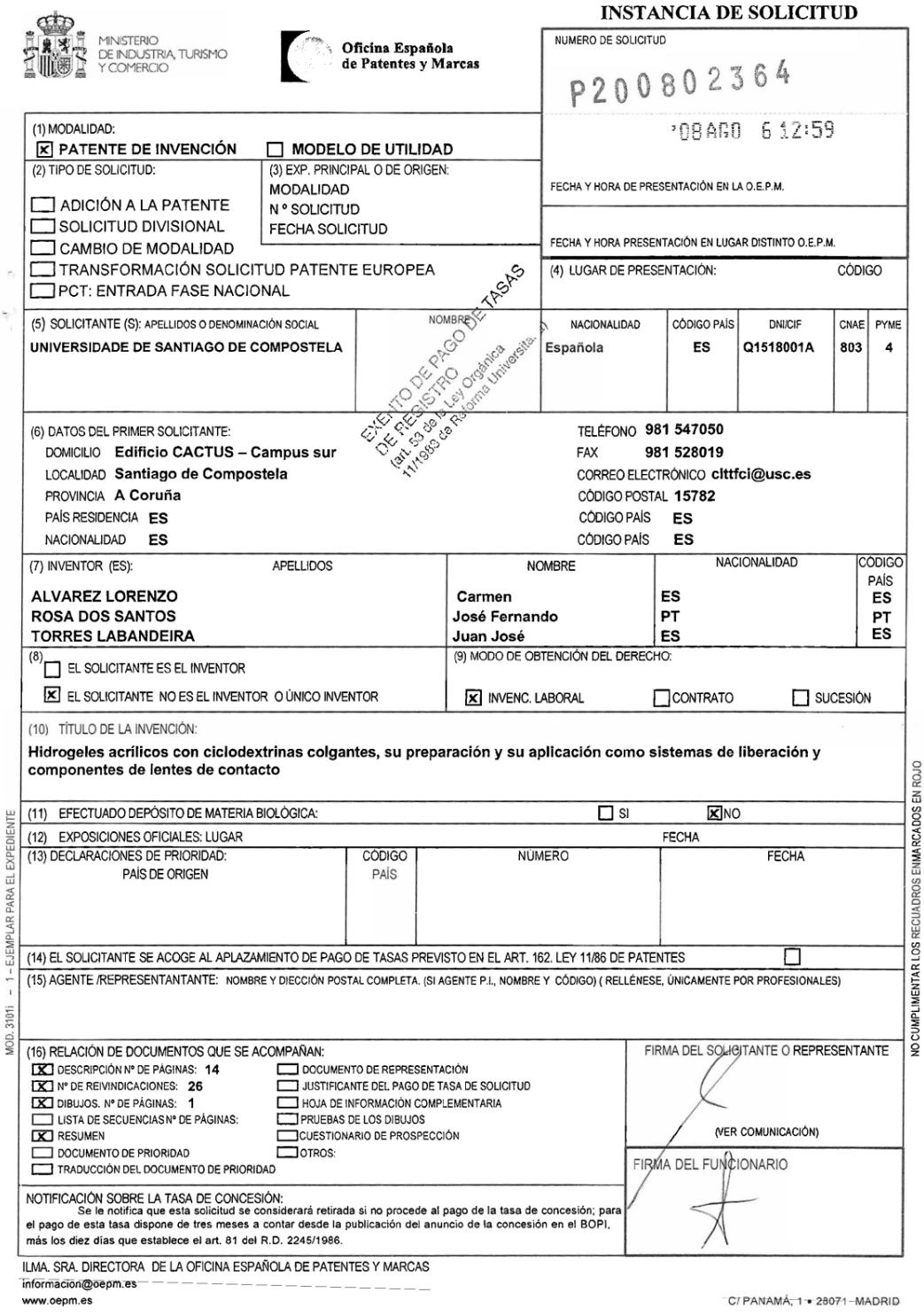
Atentamente le saluda

Fdo.: Ana López-Quiroga Valencia
Jefe Negociado de Depósito de Invenciones

Comunicación al Interesado: Los plazos de resolución se señalan en el justificante de presentación comenzarán a computarse desde la fecha de recepción que figura en la copia que se adjunta.

NOTA IMPORTANTE:
Indicación de prioridad: El código del país con el número de su solicitud de prioridad, que ha de utilizarse para la presentación de solicitudes en otros países en virtud del Convenio de París, es: **ES 200802364**

C/Castellana, 75, 1 28071 MADRID, TEL.: 902157530, FAX 913495597



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MINISTERIO DE INDUSTRIA, TURISMO Y COMERCIO

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NUMERO DE SOLICITUD: P200802364

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FECHA Y HORA DE PRESENTACIÓN EN LA O.E.P.M.

FECHA Y HORA PRESENTACIÓN EN LUGAR DISTINTO O.E.P.M.

(1) MODALIDAD:
[X] PATENTE DE INVENCIÓN [ ] MODELO DE UTILIDAD

(2) TIPO DE SOLICITUD:
[ ] ADICIÓN A LA PATENTE
[ ] SOLICITUD DIVISIONAL
[ ] CAMBIO DE MODALIDAD
[ ] TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA
[ ] PCT: ENTRADA FASE NACIONAL

(3) EXP. PRINCIPAL O DE ORIGEN:
MODALIDAD
Nº SOLICITUD
FECHA SOLICITUD

(4) LUGAR DE PRESENTACIÓN: CÓDIGO

EXENTO DE PAGO DE TASAS DE REGISTRO (art. 53 de la Ley Orgánica 11/1983 de Reforma Universita...)

(5) SOLICITANTE (S): APELLIDOS O DENOMINACIÓN SOCIAL	NOMBRE	NACIONALIDAD	CÓDIGO PAÍS	DNI/CIF	CNAE	PYME
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA		Española	ES	Q1518001A	803	4

(6) DATOS DEL PRIMER SOLICITANTE:
DOMICILIO Edificio CACTUS – Campus sur
LOCALIDAD Santiago de Compostela
PROVINCIA A Coruña
PAÍS RESIDENCIA ES
NACIONALIDAD ES
TELÉFONO 981 547050
FAX 981 528019
CORREO ELECTRÓNICO clttfci@usc.es
CÓDIGO POSTAL 15782
CÓDIGO PAÍS ES
CÓDIGO PAÍS ES

(7) INVENTOR (ES): APELLIDOS	NOMBRE	NACIONALIDAD	CÓDIGO PAÍS
ALVAREZ LORENZO	Carmen	ES	ES
ROSA DOS SANTOS	José Fernando	PT	PT
TORRES LABANDEIRA	Juan José	ES	ES

(8)
[ ] EL SOLICITANTE ES EL INVENTOR
[X] EL SOLICITANTE NO ES EL INVENTOR O ÚNICO INVENTOR

(9) MODO DE OBTENCIÓN DEL DERECHO:
[X] INVENC. LABORAL [ ] CONTRATO [ ] SUCESIÓN

(10) TÍTULO DE LA INVENCIÓN:
Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto

(11) EFECTUADO DEPÓSITO DE MATERIA BIOLÓGICA: [ ] SI [X] NO

(12) EXPOSICIONES OFICIALES: LUGAR FECHA

(13) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN	CÓDIGO PAÍS	NÚMERO	FECHA

(14) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE PAGO DE TASAS PREVISTO EN EL ART. 162. LEY 11/86 DE PATENTES [ ]

(15) AGENTE /REPRESENTANTANTE: NOMBRE Y DIECCIÓN POSTAL COMPLETA. (SI AGENTE P.I., NOMBRE Y CÓDIGO) (RELLÉNESE, ÚNICAMENTE POR PROFESIONALES)

(16) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN:
[X] DESCRIPCIÓN Nº DE PÁGINAS: 14
[X] Nº DE REIVINDICACIONES: 26
[X] DIBUJOS. Nº DE PÁGINAS: 1
[ ] LISTA DE SECUENCIAS Nº DE PÁGINAS:
[X] RESUMEN
[ ] DOCUMENTO DE PRIORIDAD
[ ] TRADUCCIÓN DEL DOCUMENTO DE PRIORIDAD
[ ] DOCUMENTO DE REPRESENTACIÓN
[ ] JUSTIFICANTE DEL PAGO DE TASA DE SOLICITUD
[ ] HOJA DE INFORMACIÓN COMPLEMENTARIA
[ ] PRUEBAS DE LOS DIBUJOS
[ ] CUESTIONARIO DE PROSPECCIÓN
[ ] OTROS:

FIRMA DEL SOLICITANTE O REPRESENTANTE
(VER COMUNICACIÓN)

FIRMA DEL FUNCIONARIO

NOTIFICACIÓN SOBRE LA TASA DE CONCESIÓN:
Se le notifica que esta solicitud se considerará retirada si no procede al pago de la tasa de concesión; para el pago de esta tasa dispone de tres meses a contar desde la publicación del anuncio de la concesión en el BOPI, más los diez días que establece el art. 81 del R.D. 2245/1986.

ILMA. SRA. DIRECTORA DE LA OFICINA ESPAÑOLA DE PATENTES Y MARCAS

información@oepm.es
www.oepm.es

C/ PANAMÁ, 1 • 28071 MADRID

MOD. 3101i – 1 – EJEMPLAR PARA EL EXPEDIENTE

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HOJA DE INFORMACION COMPLEMENTARIA

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Oficina Española de Patentes y Marcas

NÚMERO DE SOLICITUD
P200802364

FECHA DE PRESENTACIÓN

☒ PATENTE DE INVENCIÓN ☐ MODELO DE UTILIDAD

(5) SOLICITANTES: APELLIDOS O DENOMINACIÓN SOCIAL	NOMBRE	NACIONALIDAD	CÓDIGO PAÍS	DNI/CIF	CNAE	PYME
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA		ES	ES	Q1518001A	80	4

(7) INVENTORES: APELLIDOS	NOMBRE	NACIONALIDAD
CONCHEIRO NINE	Angel	ES

(12) EXPOSICIONES OFICIALES: LUGAR	FECHA

(13) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN	CÓDIGO PAÍS	NÚMERO	FECHA

MOD. 3102i – 1 – EJEMPLAR PARA EL EXPEDIENTE

NO CUMPLIMENTAR LOS RECUADROS ENMARCADOS EN ROJO

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NÚMERO DE SOLICITUD

FECHA DE PRESENTACIÓN

RESUMEN Y GRÁFICO

RESUMEN (Máx. 150 palabras)

Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto. Procedimiento de obtención de hidrogeles acrílicos con ciclodextrinas colgantes caracterizado porque los hidrogeles están constituidos por un entramado polimérico obtenido por polimerización de monómeros acrílicos o metacrílicos mono- y bifuncionales y monómeros que cuentan con grupos glicidilo en su estructura, al que una vez formado se unen covalentemente unidades de ciclodextrina; y el uso y aplicaciones de las composiciones en la preparación de lentes de contacto con capacidad para incorporar fármacos, sustancias activas o demulcentes útiles en el tratamiento de estados patológicos o fisiológicos, en la elaboración de sistemas de liberación tópica, transdérmica o transmucosal de medicamentos o sustancias activas y en la preparación de cosméticos.

GRÁFICO

(VER INFORMACIÓN)

Mod. 3103i

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(12) **SOLICITUD DE PATENTE DE INVENCIÓN**

(21) NÚMERO DE SOLICITUD

DATOS DE PRIORIDAD

(31) NÚMERO (32) FECHA (33) PAÍS

(22) FECHA DE PRESENTACIÓN

(62) PATENTE DE LA QUE ES DIVISORIA

(71) SOLICITANTE (S)

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

DOMICILIO **Edificio CACTUS - Campus sur**
15782 - Santiago de Compostela

NACIONALIDAD **Española**

(72) INVENTOR (ES)

(51) Int. Cl.

GRÁFICO (SÓLO PARA INTERPRETAR RESUMEN)

(54) TÍTULO DE LA INVENCIÓN

Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto.

(57) RESUMEN

Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto. Procedimiento de obtención de hidrogeles acrílicos con ciclodextrinas colgantes caracterizado porque los hidrogeles están constituidos por un entramado polimérico obtenido por polimerización de monómeros acrílicos o metacrílicos mono- y bifuncionales y monómeros que cuentan con grupos glicidilo en su estructura, al que una vez formado se unen covalentemente unidades de ciclodextrina; y el uso y aplicaciones de las composiciones en la preparación de lentes de contacto con capacidad para incorporar fármacos, sustancias activas o demulcentes útiles en el tratamiento de estados patológicos o fisiológicos, en la elaboración de sistemas de liberación tópica, transdérmica o transmucosal de medicamentos o sustancias activas y en la preparación de cosméticos.

Mod. 3106i

PRIMERA PÁGINA DE LA MEMORIA

Hidrogeles acrílicos con ciclodextrinas colgantes, su preparación y su aplicación como sistemas de liberación y componentes de lentes de contacto.

Sector de la técnica

Hidrogeles acrílicos con ciclodextrinas colgantes constituidos por un entramado polimérico, procedimiento de preparación de hidrogeles mediante polimerización de monómeros acrílicos o metacrílicos mono- y bifuncionales a los que una vez formados se unen covalentemente unidades de ciclodextrina; y el uso y las aplicaciones de las composiciones en la preparación de lentes de contacto con capacidad para incorporar fármacos, sustancias activas o demulcentes, útiles en el tratamiento de estados patológicos o fisiológicos, en la elaboración de sistemas de liberación tópica, transdérmica o transmucosal de medicamentos o sustancias activas y en la preparación de cosméticos.

Estado de la técnica

En los últimos años está cobrando un interés creciente el empleo de lentes de contacto blandas como reservorios capaces de liberar fármacos de manera prolongada en el área precorneal, con el fin de optimizar la biodisponibilidad ocular y hacer posible el tratamiento de patologías agudas y crónicas, aplicando pautas posológicas sencillas (Alvarez-Lorenzo et al. *Am. J. Drug Del.* 4: 131-151, 2006).

También se está prestando atención al desarrollo de lentes de contacto que contienen demulcentes para incrementar el confort durante su uso prolongado y paliar el síndrome de ojo seco (Winterton et al., *J. Biomed. Mater. Res. B* 80: 424-432, 2007; Yañez et al., *Eur. J. Pharm. Biopharm.* doi:10.1016/j.ejpb.2008.01.023).

Para llevar a la práctica la posibilidad de corregir un problema de visión y, de manera simultánea, tratar farmacológicamente una patología ocular se requieren lentes de contacto que sean capaces de incorporar fármacos en cantidades suficientes para cederlos, una vez insertadas en el ojo, a una velocidad adecuada. Entre las diversas aproximaciones que se han desarrollado para dotar de estas cualidades a las lentes de contacto blandas basadas en hidrogeles acrílicos, se cuentan la inmovilización de las moléculas de fármaco mediante la unión a través de enlaces lábiles a la estructura polimérica, la incorporación de fármaco a la lente formando parte de estructuras coloidales y la incorporación de fármaco a lentes sintetizadas utilizando monómeros funcionales, aplicando o no técnicas de moldeado molecular (molecular imprinting)

(Alvarez-Lorenzo y Concheiro, *Molecularly imprinted materials as advanced excipients for drug delivery systems*. En: *Biotechnology Annual Review* vol. 12, M.R. El-Gewely (editor), Elsevier, Amsterdam 2006, pp. 225-268).

Para obtener lentes de contacto gas permeables constituidas por entramados de ciclodextrinas, se ha propuesto la utilización de derivados lipofílicos de ciclodextrina que se reticulan mediante hidrosililación con α,ω-dihidrogeno-polidimetilsiloxano (*Patente EP 586332*). También se pueden incorporar las ciclodextrinas a lentes intraoculares y a lentes de contacto blandas formando poli-rotaxanos (complejos constituidos por polímeros lineales que se insertan en las cavidades de varias ciclodextrinas) e introduciendo, a continuación, grupos polimerizables en las unidades de ciclodextrinas de manera que puedan someterse a un proceso de polimerización que de lugar a un entramado tridimensional *(Patentes JP 2007130386; WO 2006115255; WO 2005095493; WO 2001083566)*. Estos procedimientos requieren la modificación química de las unidades de ciclodextrinas como etapa previa a la formación del entramado, lo que complica el procedimiento y puede afectar a las propiedades finales del entramado.

La incorporación de demulcentes, tales como alcohol polivinílico (PVA) o polivinilpirrolidona (PVP), se ha llevado a cabo añadiendo el demulcente libre o previa formación de un macromonómero a la mezcla de monómeros constituyentes de la lente de contacto antes de la polimerización (Bühler et al., *Chimia* 53:269, 1999; Müller B. *US Patent* 6,407,145,2002; Peterson et al., *Contact Lens Ant. Eye* 29: 127-134, 2006; Winterton et al., *J. Biomed. Mater. Res. B* 80: 424-432, 2007; Yañez et al., *Eur. J. Pharm. Biopharm.* doi:10.1016/j.ejpb.2008.01.023).

La presente invención tiene por objeto hidrogeles que son capaces de incorporar eficazmente una gran variedad de fármacos, principios activos o demulcentes mediante la formación de complejos de inclusión con ciclodextrinas colgantes.

Para incorporar ciclodextrinas a hidrogeles o entramados tridimensionales, se han desarrollado procedimientos que comprenden la síntesis de monómeros vinílicos, acrílicos o metacrílicos de ciclodextrinas y la posterior polimerización de los monómeros de ciclodextrina con otros monómeros (Lee et al., *J. Appl. Polym. Sci.* 80: 438-446, 2001; Siemoneit et al., *Int. J. Pharm.* 312: 66-74, 2006; Rosa dos Santos et al., *Acta Biomater.* 4: 745-755, 2008). Los monómeros de ciclodextrina se obtienen

haciendo reaccionar algunos de sus grupos hidroxilo o, si se trata de derivados de ciclodextrina, de sus grupos amino con monómeros que cuentan con grupos vinílico, acrílico o metacrílico. La marcha de la reacción es difícil de controlar y conduce a la obtención de monómeros que contienen grupos polimerizables en proporciones y posiciones variables. La posterior polimerización/reticulación de los monómeros da lugar a la formación de entramados en los que las unidades de ciclodextrinas son eslabones estructurales de las cadenas que constituyen el hidrogel. Ello conduce a que, en general, los entramados presenten una elevada rigidez al actuar las unidades polimerizables de ciclodextrina como agentes reticulantes. Para conseguir hidrogeles flexibles es necesario incorporar las unidades polimerizables de ciclodextrina en proporciones bajas, lo que limita la capacidad de los hidrogeles para cargar sustancias activas formando complejos de inclusión con las ciclodextrinas.

También se pueden formar hidrogeles de ciclodextrina por reticulación directa de las ciclodextrinas con agentes reticulantes que contengan dos o más grupos glicidilo en su estructura (*Patente WO 2006/089993; Rodríguez-Tenreiro et al., Pharm. Res. 23:121-130, 2006; Rodríguez-Tenreiro et al., Eur. J. Pharm. Biopharm. 66: 55-62, 2007; Rodriguez-Tenreiro et al., J. Control. Release 123: 56-66, 2007*). Esta aproximación conduce también a la formación de entramados en los que las unidades de ciclodextrinas son eslabones estructurales de las cadenas que constituyen el hidrogel.

La presente invención proporciona una solución respecto a lo conocido en el estado de la técnica que consiste en hidrogeles acrílicos con ciclodextrinas colgantes que presentan mayor capacidad para incorporar fármacos, principios activos o demulcentes y mayor capacidad para controlar su cesión. Además, estas composiciones mejoran las propiedades de resistencia mecánica, flexibilidad y estabilidad dimensional frente a la hidratación.

Descripción de la invención

En la presente invención por cadena acrílica o metacrílica se entiende una cadena polimérica que es el resultado de la polimerización de monómeros acrílicos o metacrílicos. Por unidad acrílica o metacrílica se entiende cada unidad monomérica que constituye la cadena polimérica tras la polimerización de monómeros acrílicos o metacrílicos. Las unidades acrílicas o metacrílicas monofuncionalizadas son el resultado de la polimerización de monómeros que contienen un único grupo acrílico o

metacrílico. Las unidades acrílicas o metacrílicas bifuncionalizadas son el resultado de la polimerización de monómeros que contienen dos grupos acrílicos o metacrílicos.

Un aspecto de la invención se dirige a hidrogeles en forma de red tridimensional caracterizados por estar constituidos por cadenas acrílicas o metacrílicas entrecruzadas que poseen grupos alquílicos a los que se unen ciclodextrinas mediante un enlace éter. A estos hidrogeles los denominaremos hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes, para la mejor comprensión de la memoria.

Los hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes, objeto de esta invención, presentan una elevada claridad óptica y propiedades físicas y mecánicas que las hacen útiles para su empleo como componentes de lentes de contacto blandas medicadas, de sistemas de liberación de fármacos, principios activos o demulcentes, o de cosméticos.

En un aspecto particular, las cadenas acrílicas o metacrílicas de estos hidrogeles están formadas por unidades acrílicas o metacrílicas que poseen un grupo alquiléter, unidades acrílicas o metacrílicas bifuncionalizadas y unidades acrílicas o metacrílicas monofuncionalizadas.

En un aspecto más particular, estos hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes se caracterizan porque la proporción de las unidades acrílicas o metacrílicas que contienen un grupo alquiléter está preferiblemente entre el 0,1% y el 10% en peso del hidrogel. En otro aspecto más particular, la proporción de las unidades acrílicas o metacrílicas bifuncionalizadas está preferiblemente entre el 0,1% y el 10% en peso del hidrogel.

Otro aspecto de la invención se dirige a hidrogeles en forma de red tridimensional caracterizados por estar constituidos por cadenas acrílicas o metacrílicas formadas por unidades acrílicas o metacrílicas que poseen un grupo glicidilo, unidades acrílicas o metacrílicas bifuncionalizadas y unidades acrílicas o metacrílicas monofuncionalizadas. Estos hidrogeles son materiales intermedios útiles en la preparación de hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes. A estos hidrogeles los denominaremos hidrogeles acrílicos tridimensionales con grupos glicidilo, para la mejor comprensión de la memoria.

Otro aspecto de la invención se dirige a la preparación de hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes mediante un procedimiento que

comprende la inmersión de un hidrogel acrílico tridimensional con grupos glicidilo, en una disolución de ciclodextrina a pH básico.

En un aspecto particular, los hidrogeles acrílicos tridimensionales con grupos glicidilo empleados en el procedimiento anterior, se preparan mediante la polimerización de monómeros acrílicos o metacrílicos que poseen un grupo glicidilo, monómeros acrílicos o metacrílicos monofuncionalizados y monómeros acrílicos o metacrílicos bifuncionalizados en presencia de un iniciador de polimerización.

En otro aspecto, la invención se dirige a hidrogeles para su uso como vehículo farmacéutico en la administración de un fármaco, una sustancia activa o un demulcente.

En un último aspecto, la invención se dirige al uso de hidrogeles para la elaboración de lentes de contacto que opcionalmente pueden incorporar un fármaco, una sustancia activa o un demulcente; al uso para la elaboración de sistemas de liberación tópica, transdérmica o transmucosal de un fármaco, una sustancia activa o un demulcente; y al uso para la preparación de cosméticos.

Descripción detallada de la invención

Los hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes poseen la capacidad de incorporar agua en elevadas proporciones sin disolverse, dando lugar a sistemas viscoelásticos dotados de una elevada claridad óptica.

De un modo particular, las ciclodextrinas se seleccionan preferentemente entre α-, β- o γ- ciclodextrina, una ciclodextrina compuesta por más de ocho unidades de α–1,4-glucopiranosa, o un derivado alquílico lineal o ramificado, hidroxialquílico lineal o ramificado, acetil-, propionil-, butiril-, succinil-, benzoil-, palmitil-, toluensulfonil-, acetilalquílico, glucosil-, maltosil-, carboximetil éter-, carboximetil alquil-, fosfato éster-, 3-trimetilamonio-, sulfobutil éter- ciclodextrina, o un polímero de ciclodextrina.

En un modo más particular, la proporción de ciclodextrinas está comprendida entre 1 y 0.2 unidades de ciclodextrina por cada grupo alquiléter.

Los hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes, además, tienen la capacidad de incorporar un fármaco, una sustancia activa o un demulcente. Estas composiciones son muy adecuadas para controlar la cesión de fármacos, principios activos o demulcentes. Las composiciones proporcionan velocidades de cesión diferentes dependiendo de su composición cuali- y cuantitativa y de las propiedades fisicoquímicas del fármaco, especialmente de su hidrosolubilidad y de su afinidad por la

cavidad de la ciclodextrina. Para un fármaco o una sustancia activa hidrosoluble con constante de afinidad por β-ciclodextrina igual a 170 M^{-1}, son valores típicos de porcentaje cedido un 50% al cabo de 2 días, un 80% al cabo de 8 días y un 100% al cabo de 24 días.

Las composiciones que incorporan demulcentes son útiles para reducir el coeficiente de fricción de los hidrogeles o de las lentes de contacto.

El procedimiento de obtención de hidrogeles acrílicos tridimensionales con ciclodextrinas colgantes comprende la immersión del hidrogel acrílico tridimensional con grupos glicidilo en una disolución de ciclodextrina a pH básico. De este modo, los grupos hidroxilo de las unidades de ciclodextrina reaccionan con los grupos glicidilo presentes en el hidrogel y dan lugar a enlaces éter. Si se desea, se puede proceder al lavado de los hidrogeles resultantes y opcionalmente, a su secado.

Se trata de un procedimiento ventajoso ya que no requiere la obtención previa de un monómero vinílico, acrílico o metacrílico de ciclodextrina que cuente con grupos polimerizables.

El fármaco, la sustancia activa o el demulcente se incorpora al hidrogel acrílico tridimensional con ciclodextrinas colgantes sumergiéndolo en una disolución o en una suspensión del fármaco, la sustancia activa o el demulcente. También se puede incorporar el fármaco o la sustancia activa formando complejos de inclusión con las ciclodextrinas antes de llevar a cabo la immersión del hidrogel acrílico tridimensional con grupos glicidilo.

La obtención de hidrogeles acrílicos tridimensionales con grupos glicidilo se puede llevar a cabo mediante un procedimiento que comprende la polimerización de monómeros acrílicos o metacrílicos que poseen un grupo glicidilo, monómeros acrílicos o metacrílicos monofuncionalizados y monómeros acrílicos o metacrílicos bifuncionalizados en presencia de un iniciador de polimerización.

La iniciación de la polimerización se puede realizar mediante la calefacción de la mezcla o por exposición de ésta a radiación ultravioleta-visible.

El proceso de polimerización se puede realizar en moldes de dimensiones adecuadas para dotar a los hidrogeles de la forma que se requiera para su empleo como componentes de sistemas de liberación de fármacos, principios activos o demulcentes, o como lentes de contacto medicadas.

Los hidrogeles acrílicos tridimensionales con grupos glicidilo se caracterizan por contener en su estructura unidades acrílicas o metacrílicas que poseen un grupo glicidilo, y de un modo particular los monómeros que dan lugar a estas unidades son preferentemente glicidil acrilato o glicidil metacrilato; unidades acrílicas o metacrílicas que poseen dos grupos acrílicos o metacrílicos en su estructura y que actúan como agentes reticulantes, y de un modo particular los monómeros que dan lugar a estas unidades son preferentemente etilenglicol dimetacrilato, 1,3-Butanediol diacrilato, 1,4-Butanediol diacrilato, 1,6-Hexanediol diacrilato, Etilen glicol diacrilato, Fluorescein O,O'-diacrilato, Glicerol 1,3-diglicerolato diacrilato, Pentaeritritol diacrilato monoestearato, 1,6-Hexanediol etoxilato diacrilato, 3-Hidroxi-2,2-dimetilpropil 3-hidroxi-2,2-dimetilpropionato diacrilato, Bisfenol A etoxilato diacrilato, Di(etilen glicol) diacrilato, Neopentil glicol diacrilato, Poli(etilen glicol) diacrilato, Poli(propilen glicol) diacrilato, Propilen glicol glicerolato diacrilato, Tetra(etilen glicol) diacrilato, 1,3-Butanediol dimetacrilato, 1,4-Butanediol dimetacrilato, 1,6-Hexanediol dimetacrilato, Bisfenol A dimetacrilato, Diuretano dimetacrilato, Etilen glicol dimetacrilato, Fluorescein O,O'-dimetacrilato, Glicerol dimetacrilato, Bisfenol A etoxilato dimetacrilato, Bisfenol A glicerolato dimetacrilato, Di(etilen glicol) dimetacrilato, Poli(etilen glycol) dimetacrilato, Poli(propilen glicol) dimetacrilato, Tetraetilen glycol dimetacrilato, Tri(etilen glicol) dimetacrilato, Trietilen glicol dimetacrilato, Poli(lauril metacrilato-co-etilen glycol dimetacrilato), Poli(metil metacrilato-co-etilen glicol dimetacrilato); unidades acrílicas o metacrílicas que poseen un grupo acrílico o metacrílico en su estructura, y de un modo particular los monómeros que dan lugar a estas unidades son preferentemente hidroxietil metacrilato, 1-(tristrimetilsiloxisililpropil)-metacrilato, metilmetacrilato, N,N-dimetilacrilamida, N,N-dietilacrilamida, ácido metacrílico, ácido acrílico, aminopropil metacrilato, ciclohexil metacrilato, o fluoro-siloxano acrilato.

La proporción de unidades acrílicas o metacrílicas que poseen un grupo glicidilo en su estructura está comprendida preferentemente entre el 0.1 y el 10% peso/peso del total de los componentes del entramado acrílico; la proporción de unidades acrílicas o metacrílicas bifuncionales que actúan como agentes reticulantes está comprendida preferentemente entre el 0.1% y el 10% peso/peso; y la proporción de unidades acrílicas o metacrílicas que cuentan con un grupo acrílico o metacrílico en su estructura está comprendida preferentemente entre el 80% y el 99.8% peso/peso.

La excelente biocompatibilidad de las ciclodextrinas y de los entramados acrílicos hace que las composiciones resultantes puedan ser utilizadas como componentes de dispositivos biomédicos o de lentes de contacto medicadas. Además, el procedimiento transcurre en condiciones que no comprometen la estabilidad de los fármacos, las sustancias activas o los demulcentes, y en el transcurso del proceso no se generan residuos que impliquen riesgos de contaminación ambiental.

Todo ello supone que las composiciones objeto de la invención se puedan utilizar con ventaja como componentes de sistemas de liberación tópica, transdérmica o transmucosal de fármacos o sustancias activas, como componentes de lentes de contacto medicadas con fármacos o sustancias activas o de lentes de contacto que incorporan demulcentes, y como componentes de cosméticos.

Relación de Figuras

Figura 1. Evolución en el tiempo de los valores de módulo de almacenamiento (●) y pérdida (○) del hidrogel acrílico con β-ciclodextrina colgante 1 (Tabla 1).

Figura 2. Perfiles de cesión de diclofenaco a partir de las composiciones a base de hidrogeles acrílicos con β-ciclodextrina colgante a las que se les incorporó el fármaco por inmersión en una disolución de diclofenaco sódico (Tabla 2). El ensayo se llevó a cabo introduciendo cada disco en un vial con 10 ml de una disolución acuosa de fluido lacrimal artificial de pH 8, preparada con NaCl (6.78g/L), $NaHCO_3$ (2.18g/L), KCl (1.38g/L) y $CaCl_2 \cdot 2H_2O$ (0.084g/L). Todas las composiciones controlaron el proceso de liberación hasta 24 días.

Modo de realización

A continuación, se incluyen algunos ejemplos que muestran la obtención de hidrogeles acrílicos con ciclodextrinas colgantes, la cuantificación del contenido en ciclodextrinas y la evaluación de la transparencia y de las propiedades mecánicas de las composiciones obtenidas. También, se incluyen ejemplos de la preparación de composiciones que incorporan fármacos y los liberan de manera controlada. Estos ejemplos sirven para ilustrar la invención y no son limitantes de la misma.

Para llevar a cabo la preparación de los hidrogeles acrílicos tridimensionales con ciclodextrina colgante, se prepara, en primer lugar, una disolución de ciclodextrina de concentración comprendida entre el 1 y el 5% peso/peso, en un medio constituido por disolución cloruro sódico 0.5M:dimetilformamida 50:50 v/v alcalinizada con un 3% de NaOH, utilizando un agitador mecánico o magnético y, si es necesario, aplicando

ultrasonidos. Se introduce en esta disolución el hidrogel acrílico tridimensional con grupos glicidilo, de manera que el peso de la disolución sea entre 10 y 50 veces su peso, y el sistema se mantiene a 60-90ºC durante 24 horas. Transcurrido este tiempo, los hidrogeles obtenidos se lavan con agua o con una disolución de cloruro sódico al 0.9%, a 60-90ºC o aplicando un ciclo de autoclavado. Se secan a temperatura ambiente y se sumerjen en etanol durante 24 horas reemplazando el medio cada 8 horas. El proceso de lavado se da por finalizado cuando la absorbancia del medio de lavado es menor que 0.001 en la totalidad del intervalo de longitudes de onda comprendido entre 190 y 800 nm. Los tiempos de lavado suelen estar comprendidos entre 20 minutos y 1 día. Los hidrogeles se conservan sumergidos en medio acuoso o desecados utilizando una estufa, de vacío o con corriente de aire, o aplicando liofilización.

Para cuantificar la ciclodextrina colgante en el hidrogel acrílico tridimensional con ciclodextrinas colgantes, se puede medir la absorción de moléculas orgánicas con elevada afinidad por las ciclodextrinas (typical organic compounds, TOC), tales como indol para α-ciclodextrina, ácido 3-metilbenzoico para β-ciclodextrina o rojo congo para γ-ciclodextrina (Fundueanu et al. *J. Chromatogr. B* 2003;791:407-419). El contenido final en ciclodextrinas colgantes está comprendido entre 1 y 0.2 unidades de ciclodextrina por cada grupo glicidilo presente en el entramado acrílico antes de la reacción con las ciclodextrinas. La conversión de los grupos glicidilo en enlaces éter se puede monitorizar por espectrofotometría de infrarrojos.

El fármaco o la sustancia activa se incorpora al hidrogel acrílico tridimensional con ciclodextrinas colgantes por inmersión directa del hidrogel en una disolución o en una suspensión del fármaco o de la sustancia activa, a una temperatura comprendida entre 0 y 100ºC y a la presión atmosférica, con ayuda o no de ultrasonidos. La incorporación también se puede llevar a cabo en autoclave a una temperatura comprendida entre 100 y 130ºC. También se puede incorporar el fármaco o la sustancia activa formando complejos de inclusion con las ciclodextrinas en el momento en que éstas se anclan al entramado polimérico.

El demulcente se incorpora al hidrogel acrílico con ciclodextrinas colgantes por inmersión directa del hidrogel en una disolución del demulcente, a una temperatura comprendida entre 0 y 100ºC y a la presión atmosférica, con ayuda o no de ultrasonidos. La incorporación también se puede llevar a cabo en autoclave a una temperatura comprendida entre 100 y 130ºC. Son ejemplos de demulcentes, ciertos derivados de la celulosa tales como la carboximetilcelulosa (CMC), la hidroxietilcelulosa (HEC), la

hidroxipropilcelulosa (HPC) o la metilcelulosa (MC), el dextrano, la gelatina, los polioles tales como la glicerina, el polietilenglicol (PEG), el polisorbato 80, el propilenglicol, el alcohol polivínilico (PVA) y la polivinilpirrolidona (PVP, povidona).

La cantidad de fármaco, sustancia activa o demulcente que se incorpora al hidrogel se calcula a partir de las concentraciones inicial y final de fármaco, sustancia activa o demulcente en la disolución o la suspensión utilizada para incorporarlos al hidrogel.

Las composiciones obtenidas se pueden usar para elaborar lentes de contacto que incorporen fármacos, sustancias activas o demulcentes destinadas al tratamiento de estados patológicos o fisiológicos en humanos. También se pueden utilizar para elaborar sistemas de liberación tópica, transdérmica o transmucosal de fármacos o sustancias activas y para preparar cosméticos.

Ejemplo 1. Procedimiento de obtención de un hidrogel acrílico con α-ciclodextrina, β-ciclodextrina, hidroxipropil-β-cyclodextrina, metil-β-ciclodextrina o γ-ciclodextrina colgante.

Se preparó un hidrogel de hidroxietil metacrilato (HEMA) disolviendo 0.0714 ml de etilenglicol dimetacrilato (EGDMA, 8 mM), 0.074 g de azoisobutironitrilo (AIBN, 10 mM) y 0.245 ml de glicidil metacrilato (GMA, 300 mM) en 6 ml de HEMA, inyectando la mezcla en moldes constituidos por placas de vidrio cubiertas internamente por una lámina de polipropileno y separadas por un marco de silicona de 0.4 mm de espesor, y calentando a 50ºC durante 12 horas y a 70ºC durante 24 horas más. Las láminas de hidrogel se sumergieron en agua hirviendo durante 15 minutos para eliminar los monómeros no reaccionantes y facilitar el corte de discos de 10 mm de diámetro. Los discos se sumergieron en agua durante 24 horas; a continuación, en NaCl al 0.9% durante otras 24 horas y finalmente en agua otras 24 horas más.

Los discos húmedos se sumergieron en porciones de 100 ml de disolución de α-ciclodextrina, β-ciclodextrina, hidroxipropil-β-cyclodextrina, metil-β-ciclodextrina o γ-ciclodextrina de concentración 0.019 M, que se preparó incorporando la ciclodextrina correspondiente a una mezcla de disolución acuosa de cloruro sódico 0.5 M (50 ml) y dimetilformamida (50 ml) a la que se adicionó NaOH hasta alcanzar una concentración del 3%. El sistema se mantuvo a 80ºC durante 24 horas. Los hidrogeles se lavaron con agua a 80ºC, se desecaron a temperatura ambiente, y se sumergieron en etanol 24 horas reemplazando el medio cada 8 horas.

Ejemplo 2. Procedimiento de cuantificación del contenido en ciclodextrina de un hidrogel acrílico con β-ciclodextrina, hidroxipropil-β-cyclodextrina o metil-β-ciclodextrina colgante.

Para determinar el contenido en β-ciclodextrina, hidroxipropil-β-cyclodextrina o metil-β-ciclodextrina en las composiciones de hidrogel acrílico con β-ciclodextrina, hidroxipropil-β-ciclodextrina o metil-β-ciclodextrina colgante obtenidas en el ejemplo 1, se aplicó el siguiente procedimiento. Discos secos de cada hidrogel sumergidos en disolución acuosa de ácido 3-metilbenzoico (0.5 mg/ml, 10 ml por disco) se mantuvieron durante 48 horas en la oscuridad. Transcurrido este tiempo, se determinó espectrofotométricamente a 281 nm la concentración de ácido 3-metilbenzoico y se estimó la cantidad total de ácido 3-metilbenzoico captada por el hidrogel por diferencia respecto de la concentración inicial.

La cantidad de ácido 3-metilbenzoico captada por complejación con la β-ciclodextrina, la hidroxipropil-β-cyclodextrina o la metil-β-ciclodextrina colgante se estimó sustrayendo de la cantidad total de ácido 3-metilbenzoico que captó el hidrogel acrílico con β-ciclodextrina, hidroxipropil-β-cyclodextrina o metil-β-ciclodextrina colgante, la cantidad de ácido 3-metilbenzoico captada por un hidrogel acrílico de la misma composición sin ciclodextrina.

La relación molar ciclodextrina colgante/glicidilo se situó, en todos los casos, dentro del intervalo 1-0.33.

Ejemplo 3. Procedimiento de obtención de hidrogeles acrílicos con distintos contenidos en β-ciclodextrina colgante y evaluación de sus propiedades mecánicas y ópticas.

Se prepararon hidrogeles de hidroxietil metacrilato (HEMA) disolviendo 0.0714 ml de etilenglicol dimetacrilato (EGDMA, 8 mM), 0.074 g de azoisobutironitrilo (AIBN, 10 mM) y volúmenes de glicidil metacrilato (GMA) comprendidos entre 0.041 y 0.327 ml (50 a 400 mM) en 6 ml de HEMA, inyectando las mezclas en moldes constituidos por placas de vidrio recubiertas internamente por láminas de polipropileno y separadas por un marco de silicona de 0.4 mm de espesor, y calentando a 50ºC durante 12 horas y a 70ºC durante 24 horas más. Las láminas de hidrogel se sumergieron en agua hirviendo durante 15 minutos para eliminar los monómeros no reaccionantes y facilitar el corte de

discos de 10 mm de diámetro. Los discos se sumergieron en agua durante 24 horas; a continuación, en NaCl 0.9% durante 24 horas más y finalmente en agua durante otras 24 horas.

Discos húmedos (6 discos para cada proporción de GMA) se sumergieron en porciones de 100 ml de disolución de β-ciclodextrina de concentración 0.019 M, preparada incorporando β-ciclodextrina a una mezcla de disolución acuosa de cloruro sódico 0.5 M (50 ml) y dimetilformamida (50 ml) a la que se adicionó NaOH hasta alcanzar una concentración del 3%, y se mantuvieron a 80ºC durante 24 horas Los hidrogeles se lavaron con agua a 80ºC, se desecaron a temperatura ambiente, y se mantuvieron sumergidos en etanol durante 24 horas reemplazando el etanol cada 8 horas. Finalmente se secaron en estufa a 40ºC.

Los resultados de la determinación del contenido en β-ciclodextrina, obtenidos aplicando el procedimiento que se describe en el ejemplo 2, se recogen en la tabla 1. La temperatura de transición vítrea, Tg, se determinó por calorimetría diferencial de barrido (DSC Q100, TA Instruments, USA) sometiendo 10 mg de cada hidrogel acrílico con β-ciclodextrina colgante a una rampa de calefacción desde 25ºC hasta 300ºC. Los valores de la Tg se estimaron como la temperatura que corresponde al punto medio del cambio de linea base. En la figura 1 se muestra el perfil reométrico del hidrogel 1 hidratado en agua registrado en un reómetro TA Instruments AR-1000N, aplicando una deformación del 0.5% a temperatura ambiente.

Tabla 1. Composición y temperatura de transición vítrea de hidrogeles acrílicos con β-ciclodextrina (βCD) colgante elaborados como se describe en el ejemplo 3.

Hidrogel	Proporción de GMA utilizada en la síntesis del hidrogel (mM)	GMA (mmol/g)	Contenido en βCD colgante (mmol/g)	Relación molar GMA/ βCD	Tg (ºC)
1	400	0.364	0.177	2.33	110
2	300	0.276	0.130	2.38	109
3	200	0.187	0.086	2.38	111
4	150	0.141	0.075	2.05	110
5	100	0.095	0.054	1.92	109
6	50	0.048	0.016	2.96	109
7	0	0	0	---	110

Ejemplo 4. Procedimiento de obtención de hidrogeles acrílicos con distintos contenidos en β-ciclodextrina colgante que incorporan diclofenaco sódico y lo ceden de manera controlada.

Hidrogeles de hidroxietil metacrilato con diversos contenidos en β-ciclodextrina colgante (Tabla 1) se cortaron en discos de 8 mm de diámetro y se introdujeron en viales conteniendo 10 mL de disolución de diclofenaco sódico (80 mg/L), que se mantuvieron a 25ºC. Transcurridos 4 días, se cuantificó el diclofenaco incorporado por diferencia entre la cantidad de diclofenaco presente en la disolución al inicio y al final del ensayo (valoración espectrofotométrica a 276 nm, Agilent 8453, Alemania). En la Tabla 2 se muestran, a modo de ejemplo, los contenidos en diclofenaco de discos de hidrogel de diferente composición. Los hidrogeles con mayor contenido en β-ciclodextrina colgante captaron una cantidad de diclofenaco 28 veces mayor que la que captaron los hidrogeles acrílicos de la misma composición sin β-ciclodextrina colgante.

Hidrogel	Contenido en β–ciclodextrina colgante (mmol/g)	Diclofenaco incorporado (mg/g)
1	0.177	8.6
2	0.130	7.7
3	0.086	6.4
4	0.075	4.8
5	0.054	2.6
6	0.016	2.3
7	0	0.3

Tabla 2. Cantidad de diclofenaco captado por hidrogeles con β-ciclodextrina colgante con distintos contenidos en β-ciclodextrina.

REIVINDICACIONES

1. Hidrogeles en forma de red tridimensional caracterizados por estar constituidos por cadenas acrílicas o metacrílicas entrecruzadas que poseen grupos alquílicos a los que están unidas ciclodextrinas mediante un enlace éter.

2. Hidrogeles, según la reivindicación 1, caracterizados porque las cadenas acrílicas o metacrílicas están formadas por unidades acrílicas o metacrílicas que poseen un grupo alquiléter, unidades acrílicas o metacrílicas bifuncionalizadas y unidades acrílicas o metacrílicas monofuncionalizadas.

3. Hidrogeles, según la reivindicación 2, caracterizados porque la proporción de las unidades acrílicas o metacrílicas que contienen un grupo alquiléter está preferiblemente entre el 0,1% y el 10% en peso del hidrogel.

4. Hidrogeles, según las reivindicaciones 2 y 3, caracterizados porque la proporción de las unidades acrílicas o metacrílicas bifuncionalizadas está preferiblemente entre el 0,1% y el 10% en peso del hidrogel.

5. Hidrogeles, según las reivindicaciones anteriores, caracterizados porque la ciclodextrina se selecciona preferentemente entre α-, β- o γ- ciclodextrina, una ciclodextrina compuesta por más de ocho unidades de α–1,4-glucopiranosa, o un derivado alquílico lineal o ramificado, hidroxialquílico lineal o ramificado, acetil-, propionil-, butiril-, succinil-, benzoil-, palmitil-, toluensulfonil-, acetilalquílico, glucosil-, maltosil-, carboximetil éter-, carboximetil alquil-, fosfato éster-, 3-trimetilamonio-, sulfobutil éter- ciclodextrina, o un polímero de ciclodextrina.

6. Hidrogeles, según las reivindicaciones anteriores, caracterizados porque la proporción de ciclodextrinas está comprendida entre 1 y 0.2 unidades de ciclodextrina por cada grupo alquiléter.

7. Hidrogeles según las reivindicaciones anteriores, en los que las ciclodextrinas están formando o no complejos de inclusión con fármacos o sustancias activas.

8. Hidrogeles en forma de red tridimensional caracterizados por estar constituidos por cadenas acrílicas o metacrílicas formadas por unidades acrílicas o metacrílicas que poseen un grupo glicidilo, unidades acrílicas o metacrílicas bifuncionalizadas y unidades acrílicas o metacrílicas monofuncionalizadas.

9. Hidrogeles, según la reivindicación 8, caracterizados porque la proporción de las unidades acrílicas o metacrílicas que contienen un grupo glicidilo está preferiblemente entre el 0,1% y el 10% en peso del hidrogel.

10. Hidrogeles, según las reivindicaciones 8 y 9, caracterizados porque la proporción de las unidades acrílicas o metacrílicas bifuncionalizadas está preferiblemente entre el 0,1% y el 10% en peso del hidrogel.

11. Procedimiento de obtención de hidrogeles en forma de red tridimensional, como se definieron en la reivindicación 1, que comprende la inmersión de un hidrogel, según se definió en la reivindicación 8, en una disolución de ciclodextrina a pH básico.

12. Procedimiento según la reivindicación 11, caracterizado porque la disolución de ciclodextrina tiene una concentración de entre el 1% y el 5% en peso.

13. Procedimiento según la reivindicación 11, caracterizado porque la ciclodextrina se selecciona preferentemente entre α-, β- o γ- ciclodextrina, una ciclodextrina compuesta por más de ocho unidades de α–1,4-glucopiranosa, o un derivado alquílico lineal o ramificado, hidroxialquílico lineal o ramificado, acetil-, propionil-, butiril-, succinil-, benzoil-, palmitil-, toluensulfonil-, acetilalquílico, glucosil-, maltosil-, carboximetil éter-, carboximetil alquil-, fosfato éster-, 3-trimetilamonio-, sulfobutil éter-ciclodextrina, o un polímero de ciclodextrina.

14. Procedimiento según las reivindicaciones de la 11 a la 13, caracterizado porque la ciclodextrina está formando o no complejos de inclusión con fármacos o sustancias activas.

15. Procedimiento según la reivindicación 11, caracterizado porque la inmersión del hidrogel en una disolución de ciclodextrina se mantiene a una temperatura seleccionada preferentemente entre 60 y 90ºC.

16. Procedimiento según la reivindicación 11, para la obtención del hidrogel según se definió en la reivindicación 8, que comprende la polimerización de monómeros acrílicos o metacrílicos que poseen un grupo glicidilo, monómeros acrílicos o metacrílicos monofuncionalizados y monómeros acrílicos o metacrílicos bifuncionalizados en presencia de un iniciador de polimerización.

17. Procedimiento según la reivindicación 16, en donde los monómeros acrílicos o metacrílicos que poseen un grupo glicidilo son preferentemente glicidilacrilato o glicidilmetacrilato.

18. Procedimiento según las reivindicaciones 16 y 17, en donde los monómeros acrílicos o metacrílicos bifuncionalizados se seleccionan preferentemente entre etilenglicol dimetacrilato, 1,3-Butanediol diacrilato, 1,4-Butanediol diacrilato, 1,6-Hexanediol diacrilato, Etilen glicol diacrilato, Fluorescein O,O'-diacrilato, Glicerol 1,3-diglicerolato diacrilato, Pentaeritritol diacrilato monoestearato, 1,6-Hexanediol etoxilato

diacrilato, 3-Hidroxi-2,2-dimetilpropil 3-hidroxi-2,2-dimetilpropionato diacrilato, Bisfenol A etoxilato diacrilato, Di(etilen glicol) diacrilato, Neopentil glicol diacrilato, Poli(etilen glicol) diacrilato, Poli(propilen glicol) diacrilato, Propilen glicol glicerolato diacrilato, Tetra(etilen glicol) diacrilato, 1,3-Butanediol dimetacrilato, 1,4-Butanediol dimetacrilato, 1,6-Hexanediol dimetacrilato, Bisfenol A dimetacrilato, Diuretano dimetacrilato, Etilen glicol dimetacrilato, Fluorescein O,O'-dimetacrilato, Glicerol dimetacrilato, Bisfenol A etoxilato dimetacrilato, Bisfenol A glicerolato dimetacrilato, Di(etilen glicol) dimetacrilato, Poli(etilen glycol) dimetacrilato, Poli(propilen glicol) dimetacrilato, Tetraetilen glycol dimetacrilato, Tri(etilen glicol) dimetacrilato, Trietilen glicol dimetacrilato, Poli(lauril metacrilato-co-etilen glycol dimetacrilato), Poli(metil metacrilato-co-etilen glicol dimetacrilato).

19. Procedimiento según las reivindicaciones de la 16 a la 18, en donde los monómeros acrílicos o metacrílicos monofuncionalizados se seleccionan preferentemente entre hidroxietil metacrilato, 1-(tristrimetilsiloxisililpropil)-metacrilato, metilmetacrilato, N,N-dimetilacrilamida, N,N-dietilacrilamida, ácido metacrílico, ácido acrílico, aminopropil metacrilato, ciclohexil metacrilato o fluoro-siloxano acrilato.

20. Procedimiento según la reivindicación 16, en donde la proporción de monómeros acrílicos o metacrílicos que poseen un grupo glicidilo está comprendida entre el 0,1 y el 10%.

21. Procedimiento según las reivindicaciones 16 y 20, en donde la proporción de monómeros acrílicos o metacrílicos bifuncionalizados está comprendida entre el 0,1 y el 10%.

22. Procedimiento según las reivindicaciones de la 16 a la 21, según el cual la polimerización se lleva a cabo en un molde.

23. Uso de los hidrogeles según las reivindicaciones 1 y 8, como vehículo farmacéutico en la administración de un fármaco, una sustancia activa o un demulcente.

24. Uso de los hidrogeles según las reivindicaciones 1 y 8, para la elaboración de lentes de contacto que opcionalmente pueden incorporar un fármaco, una sustancia activa o un demulcente.

25. Uso de los hidrogeles según las reivindicaciones 1 y 8, para la elaboración de sistemas de liberación tópica, transdérmica o transmucosal de fármacos, sustancias activas o demulcentes.

26. Uso de los hidrogeles según las reivindicaciones 1 y 8, para la preparación de cosméticos.

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Figura 1

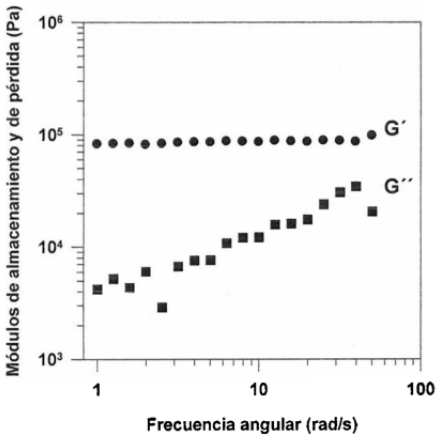
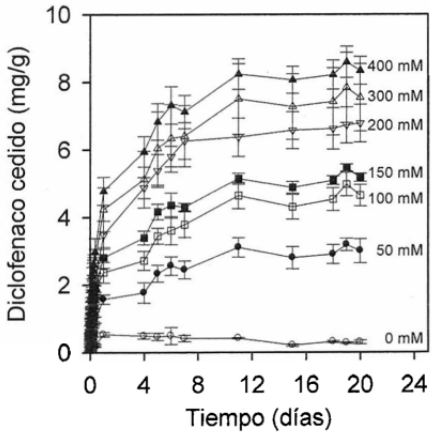


Figura 2



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Poly(hydroxyethyl methacrylate-co-methacrylated-β-cyclodextrin) hydrogels: Synthesis, cytocompatibility, mechanical properties and drug loading/release properties

Jose-Fernando Rosa dos Santos [a], Ramiro Couceiro [b], Angel Concheiro [a], Juan-Jose Torres-Labandeira [a], Carmen Alvarez-Lorenzo [a,*]

[a] *Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain*
[b] *Instituto de Ortopedia y Banco de Tejidos Musculoesqueléticos, Universidad de Santiago de Compostela, 15872-Santiago de Compostela, Spain*

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Abstract

Copolymerization of hydroxyethyl methacrylate (HEMA) with a methacrylated-derivative of β-cyclodextrin (β-CD) was evaluated as a way to obtain hydrogels with tunable mechanical and drug loading and release properties, particularly for preparing medicated soft contact lenses. A fully methacrylated β-CD monomer was synthesized and added to the HEMA and cross-linker solution at concentrations ranging from 0.042 to 0.333 g ml^{-1} (i.e. 0.23–1.82 mol.%). Thermal polymerization led to transparent hydrogels with a degree of conversion above 74%, which showed a high cytocompatibility and did not induce macrophage response. The greater the content in methacrylated β-CD was, the higher the glass transition temperature, the lower the degree of swelling and free water proportion, and the greater the storage and loss moduli of the swollen disks. These findings are directly related to the increase in the degree of cross-linking caused by the methacrylated β-CD. Loading studies were carried out with hydrocortisone and acetazolamide, both able to form complexes with CDs in water and in lacrimal fluid. Hydrocortisone loading progressively decreased as the content in methacrylated β-CD rose due to a decrease in the volume of aqueous phase of the hydrogel. Acetazolamide loading showed a maximum for an intermediate content in β-CD (0.125–0.167 g ml^{-1}) owing to a balance between complexation with β-CD and hydrogel mesh size. The hydrogels sustained drug delivery for several days, the acetazolamide release rate being dependent on the β-CD content. An adequate selection of the content in β-CD enables pHEMA-co-β-CD hydrogels suitable for specific biomedical applications to be obtained.

Keywords: Cytotoxicity; Hydrocortisone; Acetazolamide; Cyclodextrin complexation; Soft contact lenses

1. Introduction

Poly(hydroxyethyl methacrylate) (pHEMA) hydrogels are widely used as components of biomedical devices and drug delivery systems owing to their high biocompatibility, thermal and chemical stability, and tuneable mechanical properties [1–6]. The main limitation of the highly hydrophilic pHEMA materials is their poor ability to effectively load drugs and to control the release in biological medium [7,8]. Different approaches have been assayed to enhance the potential of pHEMA hydrogels as drug carriers; mainly, the chemical bound of polymerizable drugs through biodegradable links [9] and the copolymerization with functional monomers containing ionic or hydrophobic groups able to interact with the drug molecules [10–13]. Copolymerization with cyclodextrin (CD) monomers that can form inclusion complexes with drugs would be a feasible alternative.

Materials with inner microenvironments rich in CD cavities available to interact with surrounding drug molecules offer considerable possibilities for achieving an efficient

* Corresponding author. Tel.: +34 981563100x14876; fax: +34 981547148.
E-mail address: ffrusdog@usc.es (C. Alvarez-Lorenzo).

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loading and sustained release [14–21]. The attachment of CDs to a three-dimensional network hinders the fast decomplexation of the drug that usually occurs when CD–drug solutions are diluted in physiological fluids [15]. High cross-linked polymer networks, made with acrylic or vinyl monomers of CDs are particularly promising materials for controlled delivery [16,17,22–24]. For example, acrylamidomethyl-γ-cyclodextrin significantly improved the triamcinolone uptake and the control of the release from acrylic acid hydrogels [25]. The combination of HEMA with CDs has received little attention to date. Microparticles for absorbing organic pollutants from waste water or for chromatographic separations have been prepared by attachment of β-CD to preformed pHEMA microspheres using glutaraldehyde [26] or by free radical polymerization/cross-linking of HEMA with 2-hydroxy-3-methacryloyloxy-propyl-β-CD [27]. Methacrylated-β-CD monomers have also been assayed as components of highly cross-linked photopolymerizable dental composites [28,29]. However, no references to application for drug delivery have been found.

The aim of the present work was to synthesize pHEMA-co-β-CD hydrogels with low proportions of cross-linker and a wide range of contents in β-CD, and to characterize them in terms of mechanical and viscoelastic properties, interaction with water (swelling and water states), cytotoxicity, and drug loading and release performance. β-CD is poorly soluble in HEMA and, therefore, a highly substituted methacrylic monomer has to be prepared first. The biocompatibility of the resultant hydrogels was evaluated by a recently proposed method based on macrophage response to methacrylate conversion [30]. Loading studies were carried out with hydrocortisone and acetazolamide, both of practical interest for the local treatment of ocular pathologies and able to form complexes with CDs in solution, thereby increasing their hydrosolubility [31,32]. It was previously observed that Sauflon PW soft contact lenses (made with *N*-vinyl pyrrolidone and methyl methacrylate) soaked in 5% acetazolamide solution can provide sufficient concentration to lower the intraocular pressure in a more efficient way than conventional local treatments, without significant systemic absorption [33]. Since pHEMA hydrogels are receiving increasing attention as medicated soft contact lenses for ocular sustained release [7,8,34], the incidence of copolymerization with methacrylated-β-CD on the optical properties of the hydrogels and on the ability to sustain drug release in lacrimal fluid was also evaluated.

2. Materials and methods

2.1. Materials

Ophthalmic grade 2-hydroxyethyl methacrylate (HEMA) was supplied by Merck (Germany). Methacrylic anhydride, 2,6-di-tert-butyl-4-methylphenol (BHT), pyridine, 2,2′-azo-bis(isobutyronitrile) (AIBN), ethyleneglycol dimethacrylate (EGDMA), 3-methylbenzoic acid (3-MBA); acetazolamide and hydrocortisone were from Sigma–Aldrich (Spain). β-Cyclodextrin (β-CD) was supplied by Roquette-Laisa (Spain). Ultrapure water (resistivity > 18.2 MΩ cm) was obtained by reverse osmosis (MilliQ®, Millipore Spain). All other reagents were of analytical grade.

2.2. Synthesis of (2,3-di-O-methacrylated-6-methacrylated)-β-CD

A procedure based on the template polymerization method developed by Saito and Yamaguchi [35] was used. β-CD (3.6 or 7.2 g) previously dried at 105 °C for 24 h and BHT (0.04 g) were dissolved in pyridine (36 ml). Then methacrylic anhydride (19.84 g) was added, and the system stirred for 2 h at room temperature. The solution was refluxed at 50 °C for 5 h and then poured into cold water (300 ml) and stored at 4 °C overnight for precipitation of the monomer. The precipitate was filtered (0.22 μm nylon membrane, Teknokroma, Spain) and purified by dissolution in methanol (20 ml) and reprecipitation in cold water (100 ml). The purification process was repeated three more times, then the precipitate was collected and dried under vacuum. ^{1}H NMR spectra of monomer dissolved in *d*-chloroform was recorded in a Bruker AMX500 apparatus (Germany) at 500 MHz: δ[ppm] = 5.18 (C(1)H of β-CD, 7H), 4.80 (C(2)H of β-CD, 7H), 4.60 (C(3)H of β-CD, 7H), 3.58 (C(4)H of β-CD, 7H), 3.95–4.37 (C(5)H of β-CD, 7H, and C(6)H of β-CD, 14H), 5.62–6.17 (C(7)H of CH_2=C in methacrylate) and 1.95 (CH_3 in methacrylate). The number of vinyl groups per β-CD unit was estimated as the ratio of the area of C(7)H peaks of the vinyl group and the total area of protons of C(1)H [35]. FT-IR spectra of the native β-CD and the resultant monomers were recorded over the range 400–4000 cm^{-1} in a Bruker IFS 66 V FT-IR spectrometer (Germany) using the potassium bromide pellet technique.

2.3. Drug–cyclodextrin complexation in solution

Acetazolamide or hydrocortisone were added in excess to β-CD solutions (0.2–1.2% w/v) in water or artificial lacrimal fluid (6.78 g l^{-1} NaCl, 2.18 g l^{-1} $NaHCO_3$, 1.38 g l^{-1} KCl, 0.084 g l^{-1} $CaCl_2 \cdot 2H_2O$, pH 8 [36]). The resultant suspensions were kept under oscillating movement (50 oscillations min^{-1}) at 25 °C for 7 days. Then samples were taken and filtered through 0.22 μm cellulose acetate membranes (Millipore®, Spain). The concentration of dissolved acetazolamide or hydrocortisone was determined by ultraviolet spectrophotometry (Agilent 8453, Germany) at 264 or 248 nm, respectively. The apparent affinity constant of the 1:1 drug:β-CD complexes was estimated from the A_L-type diagrams using Eq. (1) [37]:

$$K_{1:1} = \frac{m}{S_0(1-m)} \quad (1)$$

where m is the slope of the plot and S_0 is drug solubility in absence of cyclodextrin.

2.4. Synthesis of pHEMA-co-β-CD hydrogels

EGDMA (0.095 ml, 80 mM, equivalent to 1.01–1.03 mol.% of total monomers) and different amounts of methacrylate-β-CD (0.25–2.0 g, 0.23–1.82 mol.%) were dissolved in HEMA (6 ml, 97.2–98.8 mol.%). Immediately after addition of AIBN (10 mM), the monomers solutions were injected into a mold constituted by two glass plates covered internally with a polypropylene sheet and separated by a 0.9 mm wide silicone frame [13]. The molds were then placed in an oven at 50 °C for 12 h and heated at 70 °C for 24 h. After polymerization, each gel sheet was immersed in boiling water for 15 min to remove unreacted monomers and to facilitate the cut of disks (10 mm in diameter). The disks were immersed in NaCl 10 mM for three days, replacing the medium each 12 h, and kept thereafter in water for a further three days. Finally, they were dried in an oven at 50 °C. Another set of hydrogels with only 8 mM EGDMA was also prepared following the same procedure.

2.5. Hydrogels characterization

2.5.1. Fourier-transformed infrared (FT-IR) analysis

FT-IR spectra of the hydrogels were obtained as described above for β-CD monomer. The FT-IR spectra of the monomers soup just before polymerization was also recorded using the ATR technique. The apparent degree of conversion of the double bonds was calculated as the reduction in the absorbance of C=C bonds at 1637 cm^{-1} normalized to the absorbance at 1724 cm^{-1}, as follows [38]:

$$DC = \left(1 - \frac{A^{\text{hydrogel}}_{1637\ cm^{-1}}/A^{\text{hydrogel}}_{1724\ cm^{-1}}}{A^{\text{monomers}}_{1637\ cm^{-1}}/A^{\text{monomers}}_{1724\ cm^{-1}}}\right) \times 100\% \quad (2)$$

2.5.2. Light transmission

The transmittance of the hydrogels at 600 nm was measured in an Agilent 8453 spectrophotometer (Germany).

2.5.3. Differential scanning calorimetry

Experiments were carried out, in duplicate, using a DSC Q100 (TA Instruments, New Castle DE, USA) with a refrigerated cooling accessory. Nitrogen was used as the purge gas at a flow rate of 50 ml min^{-1}. The calorimeter was calibrated for baseline using no pans, for cell constant and temperature using indium (melting point 156.61 °C, enthalpy of fusion 28.71 J g^{-1}), and for heat capacity using sapphire standards. To determine the glass transition temperature, T_g, the experiments were performed using non-hermetic aluminum pans, in which 5–10 mg dried disks pieces were accurately weighed, then covered with the lid and program-heated from 30 to 150 °C, cooled to 0 °C and finally heated again up to 300 °C, always at 20 °C min^{-1}. To determine the amount of freezing (unbound) water, swollen hydrogels were sealed in aluminum pans and then cooled to –30 °C and heated to 60 °C, at 5 °C min^{-1}. The total content in water of the sample (EWC) relates to the three water states (non-freezing water, W_{nf}; freezing bound water, W_{fb}; and free water, W_f) as follows:

$$EWC(\%) = W_{nf}(\%) + W_{fb}(\%) + W_f(\%) \quad (3)$$

The enthalpy of ice–water melting in the sample was proportional to the sum of W_{fb} (%) and W_f (%). The melting enthalpy of the ice formed in each sample was calculated by dividing the area of the melting peak by the total content in water of each sample. The proportion of freezing (unbound) water in the sample was estimated using as reference the melting enthalpy of ice obtained with pure water samples (330 J g^{-1}).

2.5.4. Swelling kinetics

The swelling, Q_t, of dried disks was estimated as the relative weight gain when immersed in water at 25 °C, the sample being weighed at various times t after careful wiping of its surfaces with a soft tissue:

$$Q_t = 100(W_t - W_0)/W_0 \quad (4)$$

where W_0 is the weight of the dry sample and W_t its weight at time t.

2.5.5. Viscoelasticity

The storage or elastic (G') and the loss or viscous (G'') moduli of the hydrogels, after drying and when fully swollen, were evaluated in triplicate at 25 °C, applying 0.5% strain and angular frequencies of 0.05–50 rad s^{-1} in a Rheolyst AR1000N rheometer (TA Instruments, Surrey UK) equipped with an AR2500 data analyzer, an environmental test chamber and a solid torsion kit. The sample was fixed between two clamps separated 4.7 ± 0.2 mm. Additionally, the temperature dependence of G', G'' and $\tan\delta$ ($=G''/G'$) of dried disks was recorded for 0.5% strain by measuring these parameters while increasing the temperature at 3 °C min^{-1} from 25 to 200 °C.

2.5.6. Cyclodextrin content

Dried hydrogel disks were immersed in 10 ml of 3-methylbenzoic acid (3-MBA) aqueous solution (0.5 mg ml^{-1}) and kept for 48 h at dark [39]. The concentration of 3-MBA was spectrophotometrically determined (Agilent 8453, Germany) at 281 nm. The total amount of 3-MBA taken up by the hydrogels was estimated as the difference between the initial and the final amounts in the solution. The number of functional CD cavities per gram of hydrogel was estimated by subtracting the amount of 3-MBA that can be loaded in the aqueous phase of each hydrogel or non-specifically sorbed to pHEMA from the total amount of 3-MBA loaded by the corresponding hydrogel.

2.6. Cytotoxicity

RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, VA), were maintained in Dulbecco's modified

Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10 vol.% heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD) and gentamicine (1.3 μl ml^{-1}), and kept in humidified incubator at 5% CO_2: 95% air and 37 °C. RAW 264.7 cells were seeded (5×10^5 in 2 ml) on tissue culture polystyrene 12-well plates to which sterilized hydrogel disks were added. Previously, the dried disks had been immersed in phosphate buffer (pH 7.4), stored for 4 h and autoclaved (121 °C, 20 min). Aliquots from the culture media were collected after 24 h and 5 and 10 days, and immediately frozen at −20 °C. The experiments were carried out in triplicate for each hydrogel and data point.

Cell viability was qualitatively analyzed using laser confocal fluorescence microscopy (LCS; Leica Microsystems, Germany). Samples were carefully replaced from the culture plates and washed three times with DMEM. Subsequently, adherent cells were stained with 14 mg ml^{-1} calcein-AM (Sigma–Aldrich) and 50 mg ml^{-1} propidium iodide (Molecular Probes Inc., Eugene, OR). After a two-fold washing with DMEM, the stained cells were observed under LCS and all the images obtained were processed with Leica LCS software. Cytokine concentrations (mouse IL-1α, mouse TNF-α) in cell culture supernatants were quantified by enzyme-linked immunosorbent assay (Bender MedSystems GmbH, Vienna, Austria) following the test protocol indicated by the manufacturer. The plates were read on a spectrophotometer (BioRad USA) at 450 nm. The limits of detection of mouse IL-1 α and of TNF- α were 3.1 and 4.3 pg ml^{-1}, respectively.

2.7. Drug loading and release

Dried hydrogel disks (six replicates) were placed in 10 ml of hydrocortisone solution (100 mg l^{-1}) or in 5 ml of acetazolamide solution (100 mg l^{-1}). Some systems (three replicates) were autoclaved for 20 min at 121 °C, and all were kept in the dark for four days at 25 °C. The amount loaded was estimated from the difference between the initial amount of drug in the solution and the amount remaining after loading, determined spectrophotometrically (Agilent 8453, Germany) at 248 nm for hydrocortisone or 264 nm for acetazolamide. The drug-loaded disks were rinsed with water and then immersed in 10–20 ml of artificial lacrimal fluid at 25 °C for one month. The experiments were carried out under sink conditions. The amount of drug released was measured spectrophotometrically in samples periodically taken and again placed in the same vessel so that the liquid volume was kept constant.

3. Results and discussion

3.1. Drug–cyclodextrin complexation

Since some properties of the medium, such as pH or salt concentration, may significantly alter drug complexation with cyclodextrins [40], phase solubility diagrams in lacrimal fluid were first obtained to ensure complexation in such a medium (Fig. 1). Hydrocortisone solubility diagrams were B_S-type, with sharp slopes up to a β-CD concentration of 7 mM. The $K_{1:1}$ values obtained from the linear part of the diagram were slightly altered by the ions of the medium (1.007 mM^{-1} in water vs. 1.388 mM^{-1} in lacrimal fluid). This was not the case of acetazolamide, a weak acid ($pK_a = 7.2$ [41]) with a greater solubility in lacrimal fluid than in water because of sodium salt formation. Acetazolamide solubility diagrams were A_L-type in water and B_S-type with a plateau above 8 mM β-CD in lacrimal fluid, the affinity constant being larger in water ($K_{1:1} = 0.055$ mM^{-1}) than in lacrimal fluid ($K_{1:1} = 0.022$ mM^{-1}).

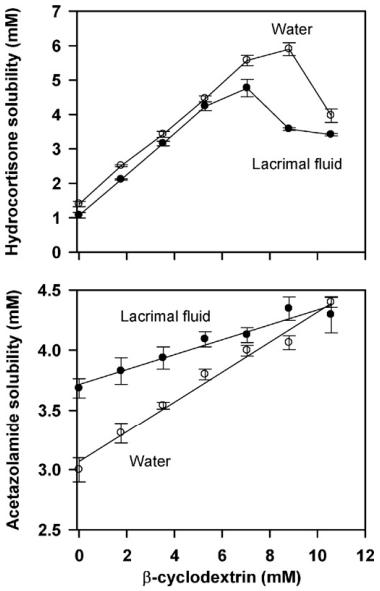

Fig. 1. Phase solubility diagrams of hydrocortisone and acetazolamide in β-cyclodextrin solutions prepared in water (open symbols) or artificial lacrimal fluid (solid symbols).

3.2. Methacrylated-β-CD monomer

To prepare (2,3-di-*O*-methacrylated-6-methacrylated)-β-CD, the concentration of methacrylic anhydride was kept constant at 2.2 M and adequate amounts of cyclodextrin were added to achieve methacrylic anhydride:β-CD molar ratios of 40 or 20. The formation of β-CD monomer was evidenced in the FT-IR spectrum by the appearance at 1720 cm^{-1} of the band characteristic of the ester groups and by an increase in absorbance at 1638 cm^{-1} due to the C=C stretching (Fig. 2). ^1H NMR analysis of the β-CD monomers confirmed that the 21 hydroxyl groups of the cyclodextrin were substituted with methacrylic groups,

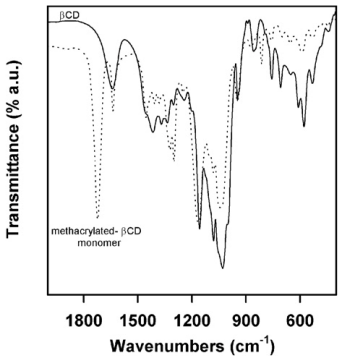

Fig. 2. FT-IR spectra of β-CD (continuous line) and its 2,3-di-*O*-methacrylated-6-methacrylated monomer (dotted line).

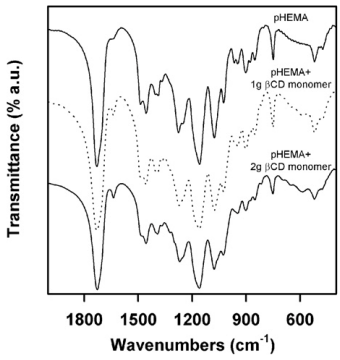

Fig. 3. FT-IR spectra of pHEMA hydrogels prepared without (upper continuous line) or with 0.167 mg ml^{-1} (dotted line) or 0.333 mg ml^{-1} (lower continuous line) 2,3-di-*O*-methacrylated-6-methacrylated-β-CD.

i.e. the (2,3-di-*O*-methacrylated-6-methacrylated)-β-CD was indeed formed whether the methacrylic anhydride:β-CD ratio in the reaction mixture was 40 or 20. Assuming a molecular weight of 2311 Da for the methacrylated monomer, the yield of the process was estimated as 85 ± 5%. The differential scanning calorimetry (DSC) scans of both the unmodified CD and CD monomer were similar, showing only an endothermic peak that is characteristic of water desorption at 100–110 °C. The temperature of decomposition of both products was above 250 °C.

3.3. Preparation and biocompatibility of Hydrogels

Unlike unmodified β-CD, the methacrylated-β-CD monomer dissolved easily in HEMA. After polymerization, the hydrogel sheets were boiled in water to remove unreacted monomers and to provide flexibility enough to cut them into small disks. The disks had a clear appearance, with a high transmittance (>90%) at 600 nm. All disks featured the bands characteristic of pHEMA: mainly, hydroxyl groups at 3330–3440 cm^{-1}, C=O amide and ester groups at 1724 cm^{-1}, ether groups at 1250–1075 cm^{-1} and a shoulder at 1022 cm^{-1} that is characteristic of β-CD (Fig. 3). The apparent degree of conversion (DC) of the C=C double bonds, estimated from 1637 cm^{-1} to 1724 cm^{-1} absorbance ratios before and after polymerization [38], was 74%. It should be noted that unmodified β-CD itself has a weak band centred at 1637 cm^{-1} (Fig. 2), which leads to an underestimation of the DC. Therefore, a 100% DC cannot be obtained using Eq. (2) and the real DC of the hydrogels is surely above 74%. This prompted us to assess the biocompatibility of the hydrogels following a recent method based on the effects of methacrylate conversion on macrophage response [30].

The dried disks were swollen in phosphate buffer (pH 7.4) and sterilized by autoclaving, and the RAW 264.7 macrophage-like cells were directly cultured on the disks for the simultaneous evaluation of leached monomers and methacrylate conversion in the polymer network. The live/dead assays carried out using calcein/ethidium staining clearly showed excellent cell viability after five days of culture (Fig. 4). In addition, it is important to note the small volume of culture medium (2 ml) in which each

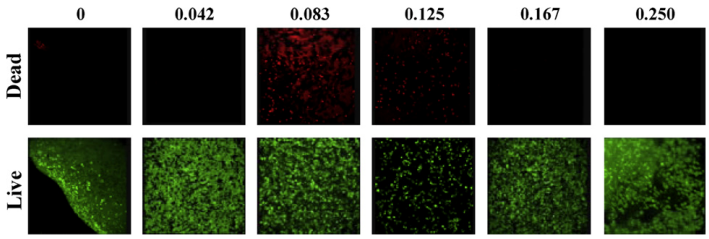

Fig. 4. Viability of RAW 264.7 cells cultured for 5 days on the hydrogel disks prepared with different proportions of β-CD monomer (mg ml^{-1} at the top of the photos). The live cells, stained with calcein AM, appear in green and the dead cells, stained with propidium iodide, appear in red. This last colorant in formulation 0.083 diffused inside the hydrogel matrix, causing a slight background red colour. Pictures for 0.333 were similar to those shown for 0.250. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrogel disk (ca. 50 mg) was immersed. If toxic monomers were leached, their concentration in the medium would be sufficient to prompt an adverse cell response, such as an inflammatory reaction. This was not the case, and the levels of the two inflammatory cytokines evaluated, IL-1 α and TNF-α, were well below the lowest standard values for all hydrogels (7.8 pg ml^{-1} for IL-1 α, and 31.3 pg ml^{-1} for TNF-α). This indicates that neither the hydrogel network nor the leachables are toxic.

3.4. Mechanical properties

The hydrogels with the highest content in β-CD were very brittle when dried. DSC scans showed that hydrogels prepared without β-CD undergo a glass-to-rubber transition at 109 °C, which is in agreement with the values previously reported for pHEMA networks [5]. Copolymerization of HEMA with methacrylated-β-CD caused a remarkable increase in the value of the glass-to-rubber transition (T_g), and when the proportion of methacrylated-β-CD was above 0.167 g per ml of monomers solution no T_g was observed below 300 °C (Table 1). Fig. 5A shows the dependence of G' and G'' on temperature for hydrogels prepared with different β-CD proportions. The higher the content in β-CD, the lower the sensitivity of the network to the temperature. Hydrogels prepared with 0.167 g or more of methacrylated-β-CD per ml remained in the glassy state up to at least 200 °C, showing high G' and G'' values. The high proportion of reactive groups enables the methacrylated-β-CD to act as an efficient cross-linker, notably increasing the cross-linking density of the network. This makes the polymeric network highly rigid. The distance between adjacent cross-linking points (i.e. the number of HEMA-mers) can be estimated as the ratio of the concentration of the main monomer, HEMA, to the sum of the concentration of cross-linking agents multiplied by their number of reactive double bonds, as follows:

$$N = [\mathrm{HEMA}]/(2[\mathrm{EGDMA}] + 21[\mathrm{methacrylated} - \beta - \mathrm{CD}])$$

If no methacrylated-β-CD is added to the monomers soup, N is 480 or 48 respectively when 8 or 80 mM EGDMA is used. Assuming that all 21 double bonds of methacrylated-β-CD react with HEMA, for 0.042, 0.083, 0.125, 0.167, 0.250 and 0.333 g of methacrylated-β-CD per ml and 8 mM EGDMA, N becomes 19.5, 9.9, 6.7, 5.0, 3.3, and 2.5, respectively. If 80 mM EGDMA is used, N decreases even more up to 14.3, 8.4, 5.9, 4.6, 3.2 and 2.4. In summary, the incorporation of methacrylated-β-CD causes a marked decrease in the length of the polymer chains between cross-linking points (at least around each cyclodextrin unit) and, as a consequence, imposes important restrictions to the movement of the network, which explains the increase in T_g.

The efficiency of methacrylated-β-CD as a cross-linker was also evident when the viscoelastic behavior of swollen hydrogels was recorded (Fig. 5B; for clarity of presentation only the plots of hydrogels with extreme behavior and only one intermediate are shown). The values of G' and G'' of the water-swollen disks were almost two orders of magnitude lower than those observed with the dried disks in the glassy state, owing to the plasticizing effect of water. G' and G'' were practically independent of angular frequency, which is characteristic of a well-structured polymer network (the slight increase in G' and G'' may be due to the loss of a small amount of water at the surface of the disks). However, it is interesting to note that, for a given EGDMA proportion, G' and G'' values progressively increased as the content in the methacrylated-β-CD of the hydrogels increased. pHEMA hydrogels without β-CD gave the lowest G' and G'' values, and the plots were superimposable for 8 and 80 mM EGDMA. By contrast, pHEMA hydrogels with a content in methacrylated-β-CD equal to or greater than 0.250 mg ml^{-1} exhibited the greatest G' and G'' values. In any case, the G' and G'' values of the fully swollen hydrogels are in the range of systems that combine the physical strength and flexibility that are required for use as soft contact lenses, implants and drug delivery platforms [42].

Table 1
Degree of swelling and amounts of 3-MBA loaded by pHEMA-co-β-CD hydrogels

Methacrylated-β-CD (g ml^{-1} of monomer solution)	EGDMA content (mM)	T_g (°C)	Swelling (%)	Free water (% referred to total water)	3-MBA loaded	
					Total (mg g^{-1} dried gel)	mol mol^{-1} CD
	80	110	60.4 (0.7)	15.5	6.18 (1.10)	
0.042	80	119	53.3 (1.6)	8.0	11.38 (0.41)	2.46 (0.18)
0.083	80	149	48.1 (2.1)	6.1	12.18 (0.11)	1.52 (0.03)
0.125	80	152	42.0 (1.1)	6.3	11.68 (0.98)	1.01 (0.16)
0.167	80		38.0 (1.3)	6.1	13.07 (0.02)	0.88 (0.01)
0.250	80		32.2 (0.8)	1.3	13.46 (1.45)	0.76 (0.02)
0.333	80		29.5 (0.6)	Not detected	10.52 (0.63)	0.42 (0.04)
	8		72.3 (1.5)		10.43 (0.11)	
0.083	8		55.8 (1.4)		14.17 (0.87)	1.05 (0.12)
0.167	8		43.2 (3.5)		14.41 (0.91)	0.68 (0.02)
0.250	8		35.9 (1.8)		15.52 (0.85)	0.63 (0.02)

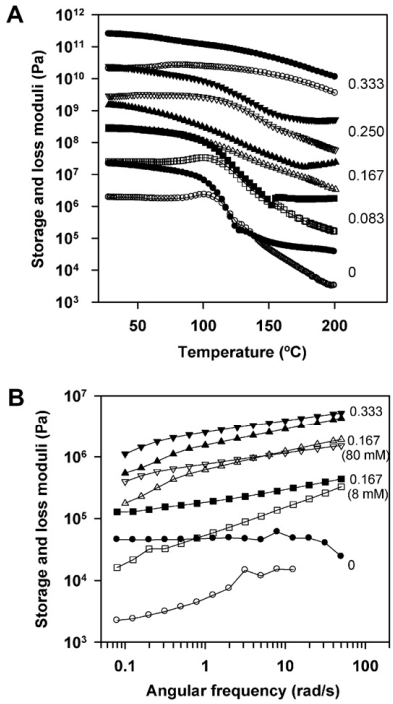

Fig. 5. Rheological properties of pHEMA hydrogels prepared with different amounts of methacrylated-β-CD (grams per ml of monomeric solution on the plot). (A) Dependence on temperature of the storage (solid symbols) and loss (open symbols) moduli of dried hydrogels for 0.5% strain. The plots corresponding to hydrogels made with 0.083, 0.167, 0.250 and 0.333 g ml^{-1} were shifted up 10, 100, 1000 and 10000 Pa, respectively, in the y-scale for a better visualization. (B) Dependence on angular frequency of the storage and loss moduli of swollen hydrogels at 25 °C. The content in cross-linker EGDMA is shown in parenthesis.

3.5. Water uptake and states of water

The uptake of water by hydrogels markedly decreased as the content in methacrylated-β-CD increased due to the increase in the degree of cross-linking and the relatively hydrophobic character of the entrapped CDs, all hydrogels reaching equilibrium within the first 4 h (Fig. 6, Table 1). Since the diffusion of solutes through a network depends not only on their content in water but on the binding state of the water, the amount of free water in the hydrogel was determined by DSC [5]. The DSC scans exhibited a single crystallization/melting peak, which was assumed to represent both free water and any freezing-bound water. As can be seen in Table 1, the greater the content in methacrylated-β-CD, the lower the proportion of free water. Hydrogels prepared with 0.250 or 0.333 g of methacrylated-β-CD per ml contained almost no free water. This may also contribute to the significantly greater G' and G'' moduli

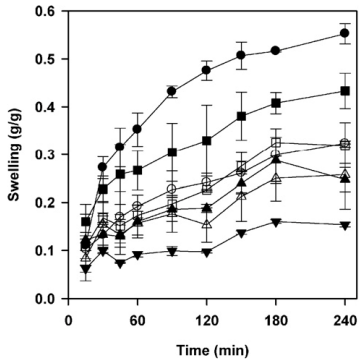

Fig. 6. Swelling profiles in water of pHEMA hydrogels prepared with 80 mM EGDMA and different amounts of methacrylated-β-CD: 0 (●), 0.042 (■), 0.083 (□), 0.125 (○), 0.167 (▲), 0.250 (△), 0.333 (▼) g ml^{-1} of monomeric solution.

recorded for these hydrogels, and could notably hinder drug diffusion during loading and release.

3.6. Content in functional β-CD units

To gain an insight into the accessibility of β-CD cavities once attached to the pHEMA network and their capability to establish inclusion complexes with small drug molecules, 3-MBA was used as a probe owing to its high affinity for β-CD ($K = 1.3 \times 10^7$ M^{-1}) [39]. Control pHEMA hydrogels loaded a significant amount of 3-MBA (Table 1), which is mainly located in the aqueous phase. pHEMA-co-β-CD hydrogels loaded greater amounts of 3-MBA. The number of molecules loaded per β-CD cavity was estimated from the difference between the total loading and the amounts loaded in the aqueous phase of the hydrogels. As can be seen in Table 1, there is a progressive decrease in the drug:β-CD molar ratio as the proportion of β-CD monomer increases. It is interesting to note that for β-CD monomer proportions of 0.042–0.125 g ml^{-1}, more than one 3-MBA molecule per β-CD is loaded. It has been previously reported that in a relatively apolar medium 3-MBA can form dimers, or even associations of greater order [43]. Therefore, some molecules could be hosted in the aqueous phase of the hydrogels by forming dimers with those partially introduced in the cavities of the β-CDs attached to the network. As explained above, hydrogels prepared with a β-CD monomer proportion equal to or greater than 0.167 g ml^{-1} have a smaller mesh size (i.e. a greater degree of cross-linking and a lower content in water). This can hinder the diffusion of 3-MBA in an amount enough to fulfill the complexation capability of the cyclodextrins. Additionally, the contribution of steric impediments owing to the proximity of the β-CD units in the network cannot be discarded. Nevertheless, even in those hydrogels prepared with the greatest β-CD

proportion, 42% of the cavities can participate in complexation (Table 1).

3.7. Drug loading

The amount of drug that can be loaded when a hydrogel is immersed in a drug solution principally depends on both the concentration in the soaking solution and the affinity of the drug for the network [44]. In the particular case of CDs, heating may promote drug complexation [40]. Thus some hydrogels immersed in hydrocortisone or acetazolamide aqueous solution were autoclaved to evaluate whether such thermal treatment enhanced the complexation when the β-CD is bound to the network. Four days in the loading solution were found to be enough to reach equilibrium. The loading of hydrocortisone progressively decreased as the content in methacrylated-β-CD rose, especially in 80 mM EGDMA hydrogels (Table 2). By contrast, acetazolamide loading showed a maximum for an intermediate content in β-CD (0.125–0.167 g ml^{-1} of monomer solution). These findings can be explained by the different affinity of the drugs for pHEMA and by their different molecular sizes. pHEMA networks can take up steroids, such as hydrocortisone, through non-specific interactions, such as hydrophobic and hydrogen bonding [45]. The possibility of establishing these types of interactions decreases as the content in CDs increases, and the complexation with the CDs does not compensate this decrease, probably due to the relatively low affinity constant. Additionally, the reduction of the mesh size of the network as the proportion of β-CD increases makes hydrocortisone diffusion progressively more difficult. The amount of drug that can be hosted in the aqueous phase of the hydrogel also decreases. This explains the positive correlation observed between the amount of hydrocortisone loaded and the degree of swelling of the hydrogels (Fig. 7). In the case of acetazolamide the dependence was more complex. Acetazolamide is significatively smaller than hydrocortisone (8.96 × 3.43 vs. 13.61 × 6.06 Å, estimated using CS ChemDraw Ultra® 5.0, CambridgeSoft, Cambridge, MA) and, therefore, the threshold of mesh size that hinders the diffusion should be lower. On the other hand, its relatively lower affinity for pHEMA enables the complexation with β-CD to play a relevant role in the loading. In fact, the hydrogels prepared with 80 mM EGDMA and an intermediate content in β-CD showed a twofold increase in acetazolamide loading, in respect of the hydrogels prepared without β-CD (Table 2). Nevertheless, in the conditions under which the loading was carried out, less than 10% of the β-CD-mers attached to the network were occupied.

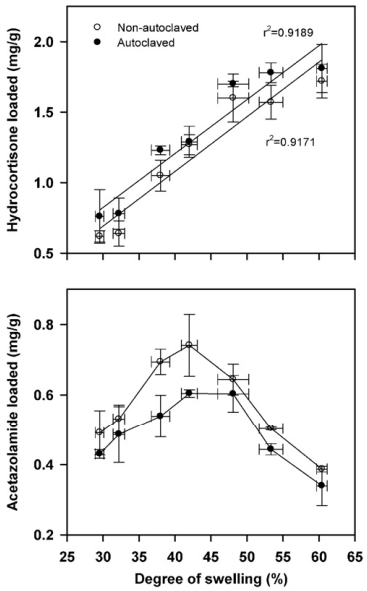

Fig. 7. Dependence of the amount of drug loaded on the degree of swelling of pHEMA hydrogels prepared with 80 mM EGDMA and different amounts of methacrylated-β-CD.

The decrease in cross-linking density due to the lower EGDMA concentration (8 mM) led to a greater loading of hydrocortisone for all β-CD proportions evaluated,

Table 2
Amounts of drug loaded by pHEMA-co-β-CD hydrogels

Methacrylated-β-CD (g ml^{-1} of monomer solution)	EGDMA content (mM)	Hydrocortisone (mg g^{-1} dried gel)		Acetazolamide (mg g^{-1} dried gel)	
		Non-autoclaved	Autoclaved	Non-autoclaved	Autoclaved
	80	1.72 (0.12)	1.81 (0.17)	0.387 (0.008)	0.339 (0.007)
0.042	80	1.57 (0.12)	1.78 (0.07)	0.503 (0.004)	0.447 (0.002)
0.083	80	1.60 (0.17)	1.70 (0.02)	0.643 (0.047)	0.615 (0.072)
0.125	80	1.27 (0.07)	1.29 (0.11)	0.740 (0.087)	0.610 (0.009)
0.167	80	1.05 (0.11)	1.23 (0.03)	0.694 (0.036)	0.597 (0.024)
0.250	80	0.64 (0.09)	0.78 (0.11)	0.491 (0.038)	0.477 (0.064)
0.333	80	0.62 (0.04)	0.76 (0.19)	0.530 (0.064)	0.430 (0.012)
	8	2.17 (0.37)	2.19 (0.26)	0.304 (0.049)	0.492 (0.018)
0.083	8	2.65 (0.37)	2.44 (0.22)	0.521 (0.065)	0.730 (0.040)
0.167	8	1.54 (0.04)	1.66 (0.38)	0.570 (0.063)	0.934 (0.039)
0.250	8	1.42 (0.17)	1.31 (0.02)	0.538 (0.076)	0.984 (0.048)

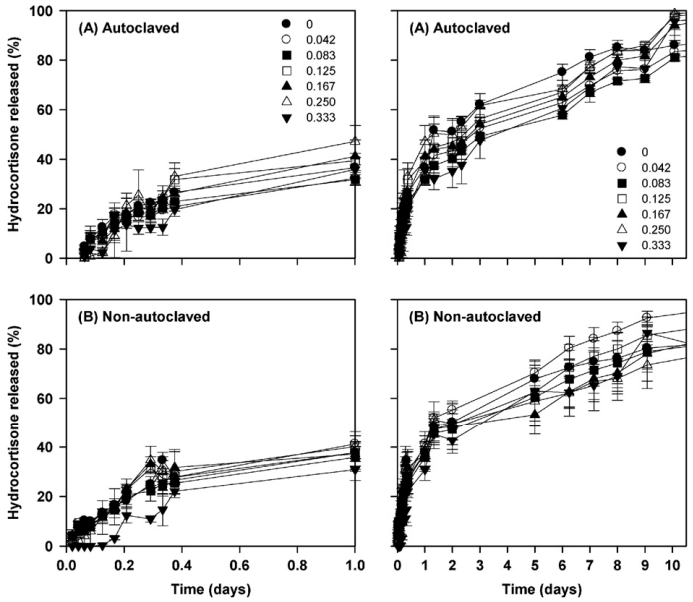

Fig. 8. Hydrocortisone release profiles from pHEMA hydrogels prepared with 80 mM EGDMA and different amounts of methacrylated-β-CD: 0 (●), 0.042 (■), 0.083 (□), 0.125 (○), 0.167 (▲), 0.250 (△), 0.333 (▼) g ml^{-1} of monomeric solution. In the interest of clarity, the short time data are shown in the plots on the left.

although, once again, the greater loadings were achieved without or with a low content in β-CD. In the case of acetazolamide, remarkable increments (up to threefold) in the loadings were only observed when the hydrogels were autoclaved to promote the complexation. Autoclaving facilitates drug diffusion inside the network and enhances the likelihood of drug–CD interaction. This effect is particularly relevant in the case of the low cross-linked hydrogels (8 mM) mainly because they could attain the loading equilibrium within the 20 min duration of the thermal treatment. This time may not be enough in the case of hydrogels cross-linked with 80 mM EGDMA. In the case of hydrocortisone, autoclaving did not enhance the loading, which confirms the minor role of the β-CDs in its loading compared with the non-specific interactions with pHEMA.

3.8. Drug release

If the drug interacts only with pHEMA, the drug release rate should be practically independent of the proportion of β-CD in the hydrogel, except where it affects the mesh size. By contrast, if drug–β-CD complexation occurs, the drug release rate would be given by the diffusion of the free drug (hosted in the aqueous phase or weakly interacting with pHEMA) and by the dissociation equilibrium of the drug–β-CD complexes. In this case, one can expect that the greater the content in β-CD, the slower the delivery.

As can be observed in Fig. 8, the hydrogels were able to sustain hydrocortisone release for at least 10 days. Despite the important differences in amount loaded, similar hydrocortisone release profiles from hydrogels cross-linked with 80 mM EGDMA were obtained and no clear dependence of release rate on β-CD content was detected. Acetazolamide release was slower and a complete delivery was attained only after 24 days (Fig. 9). The profiles were biphasic, showing an initial (first 2–3 days) relatively rapid delivery followed by a more sustained release. The greater the content in β-CD attached to the network, the slower the release rate. The most easily released drug is that which dissolves in the aqueous phase of the hydrogel or that which weakly interacts with the network. This effect predominates in pHEMA hydrogels with the lowest content in β-CD. By contrast, the greater the content in β-CD, the higher the probability of forming inclusion complexes. The prolonged sustention of the release clearly highlights the important role of the β-CDs on the interaction of acetazolamide with the network. The use of cyclodextrins enables a control of the delivery as good as or even better than that observed for contact lenses prepared by dispersing drug-loaded nanoparticles or microemulsions in pHEMA hydrogels [46].

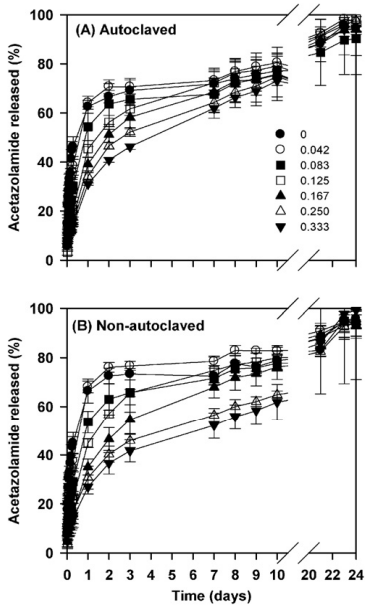

Fig. 9. Acetazolamide release profiles from pHEMA hydrogels prepared with 80 mM EGDMA different amounts of methacrylated-β-CD: 0 (●), 0.042 (■), 0.083 (□), 0.125 (○), 0.167 (▲), 0.250 (△), 0.333 (▼) g ml^{-1} of monomeric solution.

4. Conclusions

Copolymerization of HEMA with methacrylated-β-CD enables fine-tuning of the degree of swelling, mechanical properties, drug loading and release rate from hydrogels without compromising its excellent cytocompatibility. The use of a highly substituted monomer of β-CD causes an increase in the cross-linking density and in the stiffness of the hydrogel, with the consequent decrease in degree of swelling. Relatively small drugs that can diffuse inside the network and form complexes with the CDs are efficiently loaded and their release can be sustained for several days. Therefore, pHEMA-co-β-CD hydrogels could be very useful for biomedical applications.

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Soft contact lenses functionalized with pendant cyclodextrins for controlled drug delivery☆

Jose-Fernando Rosa dos Santos [a], Carmen Alvarez-Lorenzo [a,*], Maite Silva [b], Luis Balsa [b], Jose Couceiro [b], Juan-Jose Torres-Labandeira [a], Angel Concheiro [a]

[a] Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain
[b] Instituto de Ortopedia y Banco de Tejidos Musculoesqueléticos, Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain

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ABSTRACT

The aim of this work was to develop acrylic hydrogels with high proportions of cyclodextrins maintaining the mechanical properties and the biocompatibility of the starting hydrogels, but notably improving their ability to load drugs and to control their release rate. Poly(hydroxyethylmethacrylate) hydrogels were prepared by copolymerization with glycidyl methacrylate (GMA) at various proportions and then β-cyclodextrin (βCD) was grafted to the network by reaction with the glycidyl groups under mild conditions. This led to networks in which the βCDs form no part of the structural chains but they are hanging on 2–3 ether bonds through the hydroxyl groups. The pendant βCDs did not modify the light transmittance, glass transition temperature, swelling degree, viscoelasticity, oxygen permeability, or surface contact angle of the hydrogels, but decreased their friction coefficient by 50% and improved diclofenac loading by 1300% and enhanced drug affinity 15-fold. The hydrogels were able to prevent drug leakage to a common conservation liquid for soft contact lenses (SCLs) and to sustain drug delivery in lacrimal fluid for two weeks. To summarize, the hydrogels with pendant βCDs are particularly useful for the development of cytocompatible medicated implants or biomedical devices, such as drug-loaded SCLs.

1. Introduction

Medicated contact lenses are attracting escalating interest because their ability to prolong the residence time of drugs on the precorneal area, increasing the ocular bioavailability and diminishing loss by blinking and unproductive systemic absorption [1–3]. Several approaches have been assayed to enhance the drug loading capability and the controlled release efficiency of soft contact lenses (SCLs), such as drug immobilization via labile bonds [4], dispersion in the acrylic matrix or immobilization on the surface of drug-loaded colloidal nanoparticles [5–11], incorporation of functional monomers able to attract a target drug [12–14] and creation of high affinity binding receptors through molecular imprinting [15–22]. A more versatile and easily scalable alternative may be the incorporation of cyclodextrins (CDs) to the hydrogel structure. CDs can form inclusion complexes with a number of drugs through reversible non-covalent interactions. In general, the stronger the affinity constant of the drug:CD complexes, the slower the dissociation kinetics [23]. However, when solutions of drug:CD complexes are diluted in the physiological fluids, the decomplexation is practically instantaneous and controlled release cannot be achieved; such is the case of ophthalmic solutions containing CDs [24,25]. By contrast, if the CDs are attached to a polymeric network, the dilution is minimized and the microenvironment rich in CD cavities can release the drug with a rate that is negatively correlated to the affinity constant [26,27].

Vinyl- and acrylic-derivatives of CDs have been successfully used as comonomers for obtaining acrylic networks with enhanced ability to uptake drugs and to sustain the release [28–30]. Nevertheless, the number of polymerizable groups in each CD and, consequently, the degree of cross-linking of the network is not easy to regulate and when a high proportion of CD monomer is used, the network becomes too rigid to be useful as SCLs. Such hard composites have attracted interest as dental fillings [31]. The aim of this work was to develop, applying a new procedure, acrylic hydrogels that incorporate high proportions of CDs, maintain or even improve the features of the starting networks, and are able to perform as medicated contact lenses. The preparation method consists in synthesizing first the hydrogel and then attaching the CD molecules through a few of their hydroxyl groups. Thus, the CDs neither participate nor interfere in the network formation and consequently should not significantly alter its structural properties.

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* Corresponding author. Tel.: +34 981563100x14876; fax: +34 981547148.
E-mail address: carmen.alvarez.lorenzo@usc.es (C. Alvarez-Lorenzo).

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The philosophy behind this idea is similar to that applied to modify natural fibres and synthetic fabrics by surface grafting of monomers with glycidyl groups that react, in a second step, with CDs. The modified fibres maintain their mechanical properties and texture and can be loaded with odorizants or antifungal agents or used as reactive filters [32,33]. In our approach, glycidyl methacrylate (GMA) was copolymerized with monomers commonly used as components of hydrogels in order to provide the networks with binding points for β-cyclodextrin (βCD). A wide range of applications can be envisioned for such type of hydrogels. Medicated SCLs are particularly challenging since they should fulfil strict requirements as medical devices compatible with the ocular surface, which should efficiently correct ametropy and act as drug delivery systems. Thus, several proportions of GMA were used to achieve a broad range of pendant βCD proportions in hydrogels suitable for SCLs. The mechanical, optical and surface properties of the hydrogels, their cytocompatibility, oxygen permeability and diclofenac loading and sustained release ability were evaluated before and after attaching βCDs. Presently nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, are being used for the treatment of ocular inflammatory disorders (such as chronic non-infectious inflammations or seasonal allergic conjunctivitis) usually formulated in CD solutions to avoid drug precipitation and minimize its ocular irritation potential [34]. NSAIDs are also useful in decreasing bacterial colonization of contact lenses and preventing bacterial adhesion to human corneal epithelial cells [35]. Sustained delivery of diclofenac from SCLs can be a patient-friendly way of prolonging the pre-corneal retention time, improving the ocular tolerance and enhancing its therapeutic effect.

2. Materials and methods

2.1. Materials

Ophthalmic grade 2-hydroxyethyl methacrylate (HEMA) was supplied by Merck (Germany). Ethyleneglycol dimethacrylate (EGDMA), glycidyl methacrylate (GMA), 2,2′-azo-bis(isobutyronitrile) (AIBN), and 3-methylbenzoic acid (3-MBA) were from Sigma–Aldrich (Spain). β-Cyclodextrin (βCD) was supplied by Roquette-Laisa (Spain). Ultrapure water (resistivity > 18.2 MOhm cm) was obtained by reverse osmosis (MilliQ®, Millipore Spain). Diclofenac sodium was from Vorquimica S.L. (Spain). Multi-purpose solution for storage of soft contact lenses MeniCare™ Soft was provided by Menicon Co., Ltd. (Nagoya, Japan). All other reagents were of analytical grade.

2.2. Synthesis of pHEMA hydrogels with pendant cyclodextrins

EGDMA (0.0714 ml, 8 mM) and AIBN (0.074 g, 10 mM) were dissolved in HEMA (45 ml). The solution was divided into 6 ml portions to which different amounts of GMA (0, 0.041, 0.082, 0.123, 0.164, 0.245, 0.327 ml) were added to obtain a final concentration of 0, 50, 100, 150, 200, 300, and 400 mM. After few minutes of mixing, the solutions were injected into moulds constituted by two glass plates covered internally with a polypropylene sheet and separated by a silicone frame 0.9 mm thickness [15]. The moulds were then placed in an oven at 50 °C for 12 h and then heated at 70 °C for 24 h more. After polymerization, each gel sheet was immersed in boiling water for 15 min to remove unreacted monomers and to facilitate the cut of discs (10 mm in diameter). The discs were immersed in water for 24 h, then in NaCl 0.9% for 24 h more and finally stored in water. The wet discs were immersed in 100 ml of dimethylformamide: 0.5 M NaCl aqueous solution 50:50 v/v mixture containing 2.2 g βCD and 3.0 g NaOH, and kept at 80 °C for 24 h. Then the hydrogels were washed as follows: immersion in water at 80 °C for 5 min (five cycles), immersion in water at 60 °C for 24 h (three times), drying at room temperature for 24 h, immersion in ethanol (96%) for 24 h (three times) and drying at room temperature for 48 h. Some hydrogel discs were stored at the dried state and some other in USP pH 7.4 phosphate buffer until test.

2.3. FTIR analysis

FTIR spectra of the hydrogels were recorded over the range 400–4000 cm^{-1}, in a Bruker IFS 66V FT-IR spectrometer (Germany) using the potassium bromide pellet technique.

2.4. Content in βCD

Dried hydrogel discs were immersed in 10 ml of 3-methylbenzoic acid (3-MBA) aqueous solution (0.5 mg/ml) and kept for 48 h in the dark [36]. The concentration of 3-MBA was spectrophotometrically determined (Agilent 8453, Germany) at 281 nm. The total amount of 3-MBA taken up by the hydrogels was calculated as the difference between the initial and the final amounts in the solution. The number of functional βCD cavities per gram of hydrogel was estimated from the amount of 3-MBA that was loaded by the βCDs (i.e., the total amount of 3-MBA loaded by the hydrogel less the amount of 3-MBA loaded in the aqueous phase or non-specifically sorbed to pHEMA). The experiments were carried out in triplicate.

2.5. Light transmission

The transmittance of the fully hydrated hydrogels at 600 nm was measured, in duplicate, in an Agilent 8453 spectrophotometer (Germany).

2.6. Glass transition temperature

Differential scanning calorimetry (DSC) experiments were carried out, in duplicate, using a DSC Q100 (TA Instruments, New Castle DE, USA) with a refrigerated cooling accessory. Nitrogen was used as purge gas at a flow rate of 50 ml/min. The calorimeter was calibrated for baseline using no pans, for cell constant and temperature using indium (melting point 156.61 °C, enthalpy of fusion 28.71 J/g), and for heat capacity using sapphire standards. To determine the glass transition temperature, T_g, 10–11 mg dried discs pieces were accurately weighed in aluminium pans and program-heated from 30 °C to 300 °C, then cooled to 0 °C, and finally heated again up to 300 °C; always at 10 °C/min.

2.7. Swelling kinetics

Swelling, Q_t, of dried discs (three replicates) was estimated as the relative weight gain when immersed in water at 25 °C; the sample being weighed at various times t after careful wiping of its surface with a soft tissue:

$$Q_t = 100(W_t - W_0)/W_0 \quad (1)$$

where W_0 is the weight of the dry sample and W_t is the weight at time t.

2.8. Viscoelasticity

The storage or elastic (G') and the loss or viscous (G'') moduli of swollen hydrogels (two replicates) were evaluated in duplicate at 25 °C, applying 0.5% strain and angular frequencies of 0.05–50 rad/s in a Rheolyst AR1000N rheometer (TA Instruments, Surrey UK) equipped with an AR2500 data analyzer, an environmental test chamber and a solid torsion kit. The sample was fixed between two clamps separated 4.7 ± 0.2 mm.

2.9. Friction coefficient

The friction force of swollen discs was measured, in duplicate, at 35 °C using a Rheolyst AR1000N rheometer (TA Instruments, Crawley, UK) equipped with an AR2500 data analyzer and a Peltier plate [37,38]. The surface of the disc was blotted with filter paper and immediately glued (Loctite® Super Glue-3, Henkel, Barcelona, Spain) to a 4 cm steel plate geometry. 1 ml of water was put on the surface of the Peltier plate and the geometry was moved towards the plate to an initial gap of 1.0 mm. The experiment consisted of a conditioning step applying 5 ± 0.01 N normal force (W) for 15 min and a peak hold step with an angular velocity of 0.05 rad/s for other 15 min. Since the velocity changes with the distance from the center of the axis, the obtained torque, T, is a total value over the velocity range from 0 to ωR, where R is the radius of the gel disc. Thus, the total friction, F, and the coefficient of friction, μ, were determined as follows [39]:

$$F = \frac{4T}{3R} \quad (2)$$

$$\mu = \frac{F}{W} \quad (3)$$

2.10. Oxygen permeability

The oxygen permeability of the hydrogels swollen in 0.9% NaCl was measured, in duplicate, using the Createch permeometer model 210T (Rehder Development Company, Castro Valley, CA USA) fitted with a flat cell and keeping the polarographic cell in a box at near 100% relative humidity.

2.11. Surface contact angle measurements

The water contact angle on dried hydrogel discs (in duplicate) was measured in static mode using an OCA 15 plus video based optical contact angle measuring device fitted with software SCA 20 (Data Physics Instruments GmbH, Germany). A

drop of 10 μl water was deposited on the hydrogel disc and the contact angle was measured at several times for 6 min.

2.12. Cytocompatibility

RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, VA), were maintained in GIBCO™ Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% v/v heat inactivated fetal bovine serum (Life Technologies, Rockville, MD) and gentamicine (130 μl/100 ml), and kept in humidified incubator at 5% CO_2: 95% air and 37 °C. RAW 264.7 cells were seeded (2×10^5/2 ml) on tissue culture polystyrene 24-well plates to which sterilized hydrogel discs were added. Previously, the dried discs were immersed in phosphate buffer pH 7.4, stored for 4 h, and autoclaved (121 °C, 20 min). Aliquots from the culture media (D-MEM with heat inactivated fetal bovine serum and gentamicine, as described above) were collected after 24 h and 4 and 8 days, and immediately frozen at −20 °C. The experiments were carried in triplicate for each hydrogel and data point.

Cell viability was qualitatively analyzed using Laser Confocal Fluorescence Microscopy (LCS, Leica Microsystems, Germany). Samples were carefully replaced from the culture plates and washed three times with D-MEM. Subsequently, adherent cells were stained with 1 mg/ml Calcein-AM (Sigma–Aldrich, Spain) and 1 mg/ml propidium iodide (Molecular Probes Inc., Eugene, OR, USA). After a two-fold washing with D-MEM, the stained cells were observed under LCS and all the images obtained were processed with Leica LCS software. Cytokine concentrations (mouse IL-1α, mouse TNF-α) in cell culture supernatants were quantified by specific enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems GmbH, Austria) following the test protocol indicated by the manufacturer. The plates were read by a spectrophotometer (BioRad USA) at 450 nm. The limits of detection of mouse IL-1α and of TNF-α were 7.8 pg/ml and 31.8 pg/ml, respectively. Hydrogels of pHEMA without GMA were used as controls of recognized cytocompatibility for the tests of pHEMA-*co*-GMA hydrogels and of the hydrogels with pendant βCD. In each case, the control hydrogels underwent the same treatments as the hydrogels under evaluation.

2.13. Diclofenac loading

Dried hydrogel discs (six replicates) were placed in 10 ml of diclofenac sodium aqueous solution (80 mg/l) and kept four days at 25 °C protected from light. The amount loaded was estimated from the difference between the initial amount of drug in the solution and the amount remaining after loading, determined spectrophotometrically (Agilent 8453, Germany) at 276 nm.

2.14. Storage test

Some drug-loaded discs were placed in lens cases filled with 1 ml of multipurpose solution for soft contact lenses and stored for one month protected from light at room temperature. The leakage of diclofenac to the medium was quantified spectrophotometrically. The experiments were carried out in triplicate.

2.15. Diclofenac release

Diclofenac release was evaluated both after immediate loading of the lenses and after storage in the multipurpose solution. In both cases, the release experiment was carried out first by rinsing of the lenses with water, followed by immersion in 10–20 ml of artificial lacrimal fluid at 25 °C. The experiments were carried out in triplicate under sink conditions. The amount of drug released was measured spectrophotometrically in samples (1 ml) periodically taken and again placed in the same vessel so that the liquid volume was kept constant.

3. Results and discussion

3.1. Hydrogel structure

The pHEMA hydrogels were prepared following the conventional approach of free radical polymerization, but using GMA as comonomer at several proportions (Table 1) in order to have glycidyl groups both in the bulk and at the surface of the hydrogel. After polymerization and before the grafting of βCDs, the hydrogel sheets were boiled in water to remove the unreacted monomers and to make it easier to cut them into small discs. It has been previously shown that glycidyl groups are suitable for reacting with the hydroxyl groups of native βCD under certain conditions [26]. Thus, the GMA hydrogel discs were immersed in βCD alkaline solutions mixed with several polar aprotic solvents at 80 °C. The best chemical stability of the hydrogels (hydrolysis of ester groups) and the highest yield of binding of βCDs were obtained using dimethylformamide as cosolvent. An optimized washing protocol for the complete extraction of dimethylformamide from the βCD cavities was then applied. This protocol also ensured the removal of free βCDs.

Table 1
Composition of the hydrogels upon synthesis, amount of 3-MBA uptaken by the hydrogels before and after grafting of βCD, and available cavities, molar ratio of GMA/βCD, and glass transition temperatures of the hydrogels with pendant βCD.

Hydrogel	[GMA] (mM)	GMA (mmol/g)	3-MBA sorbed before treatment with βCD (mg/g)	3-MBA sorbed after treatment with βCD (mg/g)	Available βCD cavities (mmol/g)	GMA/βCD (mol ratio)	T_g (°C)
SCL1	400	0.364	13.57 (0.93)	37.63 (1.70)	0.177	2.33	110
SCL2	300	0.276	13.04 (1.18)	30.72 (0.86)	0.130	2.38	109
SCL3	200	0.187	11.88 (0.51)	24.63 (3.14)	0.086	2.38	111
SCL4	150	0.141	12.63 (0.18)	22.88 (0.59)	0.075	2.05	110
SCL5	100	0.095	11.77 (0.68)	19.08 (1.19)	0.054	1.92	109
SCL6	50	0.048	12.77 (0.34)	14.97 (0.34)	0.016	2.96	109
SCL7	0	0	12.98 (0.38)	13.11 (0.15)	–	–	110

All hydrogels with pendant βCD showed a smooth surface (similar to the unmodified hydrogels as observed using environmental scanning electron microscopy) and a high transparency with a transmittance above 90% at 600 nm. FTIR spectra were very similar for both the hydrogels before and after the treatment with βCD (Fig. 1) which can be attributed to that the characteristics bands of the glycidyl groups (1120 cm^{-1}) and of βCD overlapped with those of the numerous ether groups of the pHEMA-based hydrogel. Therefore, an indirect method to confirm the grafting of βCD was applied. Such a method based on the typical organic compound (TOC) approach involves the use of 3-MBA as a probe with a high affinity for βCD (1.3×10^7 M^{-1}) [30,36]. The amount of βCD per hydrogel weight unit was quantified in terms of βCD able to form inclusion complexes with 3-MBA. pHEMA hydrogels without βCD loaded a small amount of 3-MBA (Table 1), which may be mainly located in the aqueous phase. Since no influence of GMA content in the loading of 3-MBA was observed, chemical interactions between the probe and the glycidyl groups can be discarded.

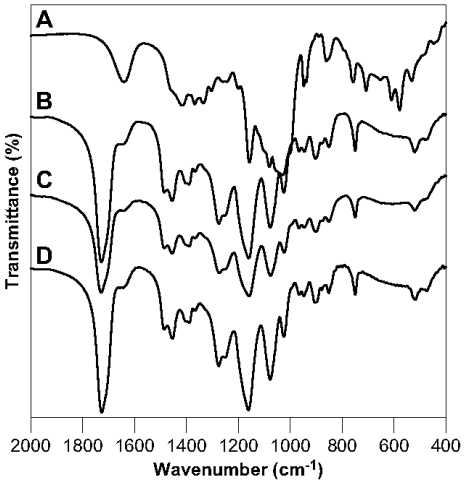

Fig. 1. FTIR spectra of βCD (A), pHEMA-*co*-GMA (400 mM) hydrogel before (B) and after grafting βCD (C) and control pHEMA hydrogel (D).

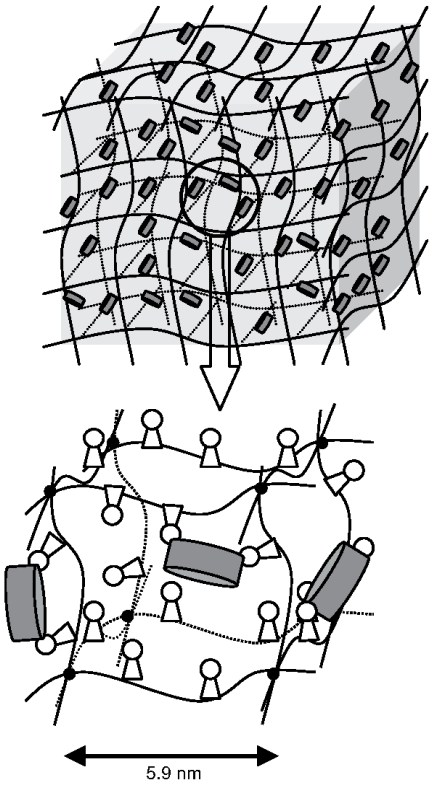

Fig. 2. Scheme of a pHEMA-*co*-GMA hydrogel with pendant βCDs.

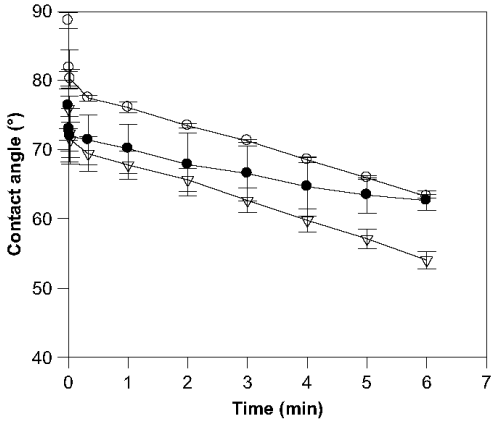

Fig. 3. Time evolution of the contact angle of control pHEMA hydrogel (down triangles) and pHEMA-*co*-GMA (400 mM) hydrogel before (open circles) and after (full circles) grafting βCD. The error bars represent the standard deviations of measurements carried out in duplicate.

Hydrogels with pendant βCD showed an order correlation between the loading of 3-MBA and the proportion of GMA units upon synthesis, which clearly indicates that the glycidyl groups have reacted with the βCD units. However, the number of βCDs per gram of hydrogel was 2–3 times lower than that of GMA groups (Table 1). This finding is explained taking into account the mesh size, the distance among GMA units and the dimensions of the βCD, as follows. Upon synthesis, the distance between adjacent cross-linking points was estimated from the number of molecules of cross-linker EGDMA per unit of volume (cm^3) of hydrogel [40]:

$$R_X = \frac{10}{\sqrt[3]{[\mathrm{EGDMA}]N_A}} \quad (4)$$

N_A being the Avogadro's number. In our case, R_X equals to 5.92 nm; i.e. assuming that the hydrogel is formed by cubic cells, the length of each side would be 5.92 nm. This estimation is for non-swollen conditions. When hydrogel swells in water (water content around 60%, as discussed below), the volume of the swollen hydrogel is around 150% that of the anhydrous gel, which increases the mesh

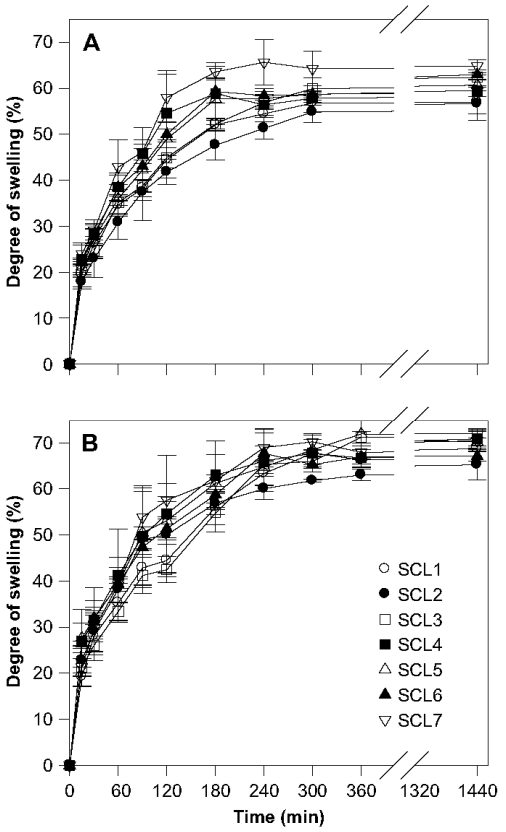

Fig. 4. Swelling profiles of the hydrogels before (A) and after (B) grafting of βCD. Codes as in Table 1. The error bars represent the standard deviations of measurements carried out in triplicate.

size up to 6.72 nm. Since the diameter of the widest side of βCD is 1.53 nm and the depth of the cavity is 0.78 nm [24], no steric hindrance for the diffusion of this cyclodextrin through the mesh of the hydrogel is expected. Similarly, the average distance between GMA groups was estimated from the number of GMA mers per unit of volume as follows:

$$R_{GMA} = \frac{10}{\sqrt[3]{[GMA]N_A}} \quad (5)$$

The values ranged from 3.2 nm, for the hydrogel prepared with 50 mM GMA, to 1.6 nm for the hydrogel prepared with 400 mM. These distances (a 14% greater when the hydrogel is fully swollen), together with the length of the glycidyl group and the flexibility of the low cross-linked network, makes the reaction of each βCD with 2 or even 3 glycidyl groups feasible (Fig. 2).

3.2. Mechanical, surface properties and swelling

Pendant βCDs did not significantly alter the glass transition temperature (T_g) of the hydrogels (Table 1), which means that the grafted βCDs did not increase the cross-linking degree of the hydrogels nor the stiffness of the network. This finding contrasts with the features of pHEMA-*co*-βCD hydrogels obtained using (2,3-di-O-methacrylated-6-methacrylated)-β-CD as comonomer, which showed a rapid increase in T_g as the content in βCD increased. Above 0.6 mol% such hydrogels had a T_g beyond 150 °C, thus being too rigid for preparing SCLs [30]. By contrast, the hydrogels developed in the present work can contain at least 2.5 mol% βCD without increasing the stiffness, which may also improve the availability of the βCD cavities to host drug molecules.

The wettability of hydrogels surface is a critical variable when used as components of SCL since it affects their physiological compatibility and the stability of the pre-lens lacrimal fluid [41].

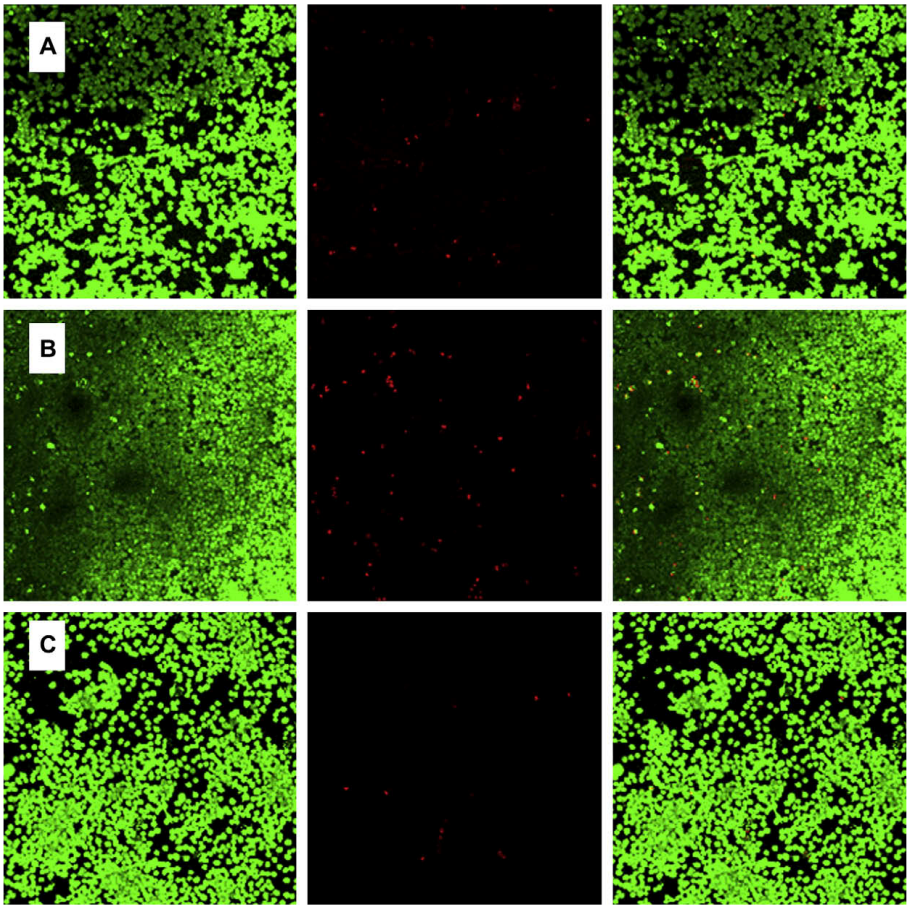

Fig. 5. Viability of RAW 264.7 cells cultured for 5 days on pHEMA hydrogels without GMA nor βCD (A), hydrogels with the intact GMA groups (400 mM, B) and hydrogels with pendant βCDs (C). The live cells stained with calcein AM appear in green (left panel) and the dead cells stained with propidium iodide appear in red (in the middle). Simultaneous staining appears at the right panel.

The hydrophilicity of the surface of pHEMA-dried hydrogels was evaluated through contact angle measurements. The contact angle slightly increased when copolymerized with high proportions of GMA, but slightly decreased again after the βCD were attached (Fig. 3). Overall, βCD had a favorable effect on the wettability. Once immersed in water, all hydrogels reached the equilibrium of swelling in 4–5 h. Although the degree of swelling was quite similar for all hydrogels, the presence of GMA made the networks slightly more hydrophilic and βCD did not cause relevant changes on the swelling of the network (Fig. 4).

In addition to wettability and water content, the comfort of SCL wearers also depends on the viscoelasticity of the network and on the sliding on eye surface and the lids. The storage and loss moduli at 5 rad/s of the hydrogels with the highest content in βCD were 80 and 10 KPa, respectively, which are in the range considered appropriate for SCL combining comfort and visual performance with the required physical strength [42]. The friction coefficients of the new hydrogels were estimated using rheometry, which is an advantageous approach compared to tribometry mainly because the greater sensitivity and the more precise control of the temperature of the sample and of the liquid trapped between the geometry and the solid surface (Peltier plate) [37,39]. The friction experiments were carried out at 35 °C to simulate the temperature of the ocular surface. pHEMA hydrogels with unmodified GMA groups showed values of friction coefficient, μ, against the Peltier plate (surface of chrome-plated copper) of 0.35 ± 0.05, which is in the range of the values previously reported for pHEMA hydrogels commonly used as SCL [37] and as synthetic articular cartilage [43]. The grafting of βCD caused, in all hydrogels, a decrease in μ of around 50% ($\mu = 0.15 \pm 0.05$). The lower friction coefficients recorded after the grafting of βCD can be attributed to both a slightly greater degree of swelling and to the presence at the hydrogel surface of sliding βCD units.

3.3. Cytocompatibility

Oxygen permeability is crucial in preventing corneal hypoxia and edema during SCL wearing. Grafting of βCD did not alter the oxygen permeability of the hydrogels, being 1.22×10^{-9} (cm^2/sec) (ml O_2/ml × mmHg) or 122 barrer, which is in the range of common SCLs [44]. The variability among hydrogels differing in βCD content was below 5%. Since the main monomers used to prepare the hydrogels (HEMA and EGDMA) are common components of FDA approved contact lenses [2,45] and βCD is regarded as non-irritant to the eyes [46], good compatibility with the ocular surface is foreseeable for the hydrogels with pendant βCD. Nevertheless, the potential adverse effects of residual monomers or unreacted double bonds remaining in the hydrogel network should be tested against macrophages which are particularly sensitive to methacrylate groups [47]. The cytocompatibility studies were carried out both before and after the treatment of hydrogels with βCD. The dried discs were swollen in pH 7.4 phosphate buffer and sterilized by autoclaving, and the RAW 264.7 macrophage-like cells were directly cultured on the discs for the simultaneous evaluation of the effect of leached substances, unreacted methacrylate and the presence of βCD in the polymer network. The RAW 264.7 cells covered the surfaces of the hydrogels with pendant βCD as those of the control PHEMA and PHEMA-*co*-GMA hydrogels. The live/dead assays carried out using calcein/ethidium staining clearly showed excellent cell viability after 5 days of culture (Fig. 5) both for the hydrogels with the intact GMA groups (Fig. 5B) and the hydrogels with the pendant βCDs (Fig. 5C), and similar to that of hydrogels without GMA nor βCD (Fig. 5A). It is important to note that each hydrogel disc (ca. 50 mg) was immersed in a small volume of culture medium (2 ml) and if toxic substances were leached, their concentration in the medium could be sufficient to actuate an adverse cell response. To gain an insight into possible inflammatory reactions, the secretion of cytokines such as interleukin-1 (IL-1α) and tumour necrosis factor (TNF-α) was quantified. The levels of IL-1α, which is the triggering switch in the initiation of inflammation, were below the quantification limit (7.8 pg/ml) for all hydrogels. By contrast, all hydrogels elicited detectable TNF-α response in the first 24 h (ca. 1000 pg/ml) but the levels progressively diminished in the first 4 days. Compared to the control hydrogel prepared without GMA, neither the copolymerization with GMA nor the grafting of βCD caused relevant changes in the TNF-α levels. Our results are in agreement with previous reports which show that hydrogels with epoxy groups have a low toxicity [48,49].

3.4. Drug loading and release

Hydrogel discs were immersed into sodium diclofenac aqueous solution and their drug loading ability was quantified. Those hydrogels prepared without GMA and that consequently do not have pendant βCD could only load a small amount of diclofenac, mainly incorporated into the aqueous phase of the hydrogel and nonspecifically sorbed to the polymeric network [14]. In contrast the drug uptake markedly rose as the proportion in pendant βCDs increased (Fig. 6). The difference between the amount of drug loaded by the hydrogels with pendant βCDs and by the control one (without cyclodextrin) is due to the drug hosted by the cyclodextrin cavities.

The amount loaded by just a simple equilibrium between the aqueous phase of the network and the loading solution, which leads the drug concentration within the hydrogel to be equal to that of the loading solution, can be estimated using the following equation proposed by Kim et al. [50]:

$$\text{Loading(aqueous phase)} = (V_s/W_p) \times C_0 \quad (6)$$

where V_s is the volume of water sorbed by the hydrogel, W_p the dried hydrogel weight, and C_0 the initial concentration of drug in the loading solution. For a water uptake of 0.6 ml/g, the loading in the aqueous phase is 0.048 mg/g, which is smaller than the values

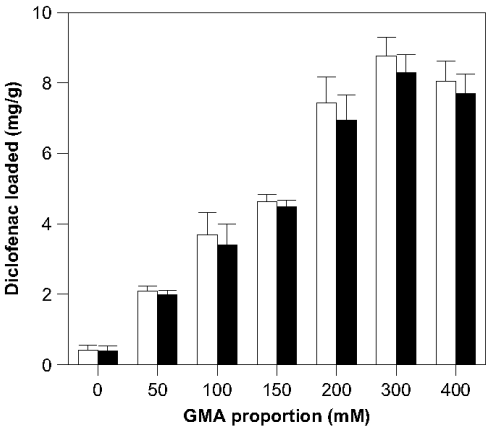

Fig. 6. Diclofenac loaded by the hydrogels after immersion in the drug solution (white columns; mean values with standard deviations, $n = 6$) and amount of diclofenac remaining in the hydrogel after storage for 30 days in contact lens fluid (black columns; mean values with standard deviations, $n = 3$).

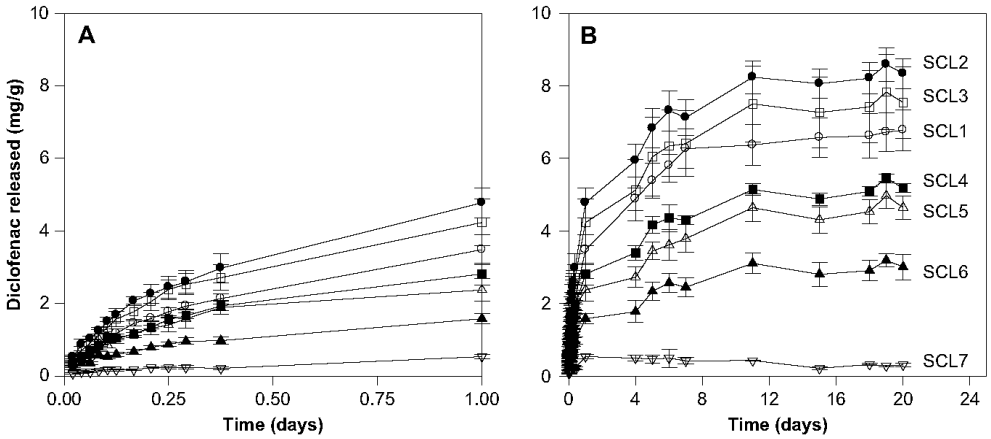

Fig. 7. Diclofenac release profiles from freshly loaded hydrogels with pendant βCDs, during the first 24 h (A) and the whole period of 20 days (B). Codes as in Table 1. The error bars represent the standard deviations of measurements carried out in triplicate.

shown in Fig. 6. This means that the drug can establish hydrophobic interactions with the network and form inclusion complexes with cyclodextrin cavities through its aromatic rings. In order to further understand the role of the interaction of diclofenac with cyclodextrin units, the partition coefficient, K, between the polymer network and the drug loading solution was estimated from the following expression [50]:

$$\text{Loading(total)} = [(V_s + KV_p)/W_p \times C_0] \quad (7)$$

where V_p is the volume of dried polymer and the other symbols maintain the same meaning as in Eq. (6). The values of K were 7, 28, 32, 59, 79, 95, and 107 for SCL7, SCL6, SCL5, SCL4, SCL3, SCL2, and SCL1, respectively. These values clearly indicate that greater the content in pendant βCDs, higher the affinity of the drug to the network. Furthermore, these loading levels corresponded to a diclofenac: βCD molar ratio of 0.20–0.25, which means that the 75–80% βCD is still available for hosting more drug. The maximum number of diclofenac molecules that each hydrogel can load is mainly given by the number of functional βCD cavities present in the hydrogel (see Table 1; e.g. SCL 1 can load per gram up to 0.177 mmol or 36.5 mg of diclofenac). Thus, greater amounts of diclofenac could be loaded in the hydrogel by increasing the drug concentration in the loading solution [26]. The mechanism that prompt the inclusion of diclofenac into the βCD cavities relays on the gain in entropy that occurs when thermodynamically unstable water inside the βCD cavity is replaced by a more hydrophobic molecule or a lipophilic moiety on. The guest molecule can establish favorable interactions with the hydrophobic inner face of the cavity and hydrogen bonds with the outer –OH groups of the cyclodextrin [23]. Any other drug possessing lipophilic groups, particularly aromatic rings, may be also a suitable candidate for being delivered using the hydrogels with pendant βCDs.

To gain an insight into the potential application of this type of hydrogels as drug-loaded SCLs, the next step was to store drug-loaded discs in a commercially available fluid used to conserve soft contact lenses (MeniCare Soft, Menicon Co. Ltd, Japan). Each disc was stored in a SCL case containing 1 ml of the conservation fluid, hermetically closed and at room temperature. After 30 days, the concentration of the drug in the fluid was spectrophotometrically measured. Drug leakage to the fluid was irrelevant as can be appreciated in Fig. 6. Therefore, the hydrogels with the pendant βCDs are able to load ample amounts of drug and to prevent the release during storage in a common conservation liquid for SCLs.

Diclofenac release from the hydrogels was evaluated in artificial lacrimal fluid using both freshly loaded discs and one month-stored discs. For each type of hydrogel, drug release rate was similar in both cases. The hydrogels without pendant βCD rapidly delivered the complete dose by diffusion, the whole process finishing within one day (Fig. 7). By contrast, the hydrogels with pendant βCDs were able to sustain the delivery for several days, even up to 2 weeks, particularly those containing high proportions of βCD (in which uncomplexation controls the release). Despite the experiments being carried out under *sink* conditions, the high affinity of the drug for the hydrogels with pendant βCDs stopped the release when a certain drug concentration in the medium was reached. This means that equilibrium between drug release and reabsorption by the hydrogels was attained. For the complete extraction of the drug, the release medium needed to be replaced. If intended to be used as drug-loaded SCL, the hydrogels could even sustain the release for a longer period of time in the reduced volume of the precorneal lacrimal fluid. It is foreseeable that the hydrogel disc delivers drug until a certain concentration is attained; the affinity for the drug preventing a burst release or achieving high local concentration. Thus, as the drug is drained from the ocular surface, more drug will be released to maintain the equilibrium concentration.

4. Conclusions

The new method is suitable for grafting βCD to pHEMA-*co*-GMA networks under mild conditions, the βCD:glycidyl group ratio being mainly 1:2 or 1:3. pHEMA hydrogels with pendant βCDs are highly biocompatible. Grafting of βCD does not significantly alter the glass transition temperature, swelling, optical transparency or oxygen permeability of the network, but decreases the friction coefficient. The pendant βCDs notably improved the ability of the hydrogels to load diclofenac and to prevent drug leakage during storage in conservation liquid for SCL. Drug release was sustained for up to two weeks in lacrimal fluid. The hydrogels with pendant βCDs have two main advantages compared to networks of copolymerized βCD monomers: i) they maintain the mechanical properties of the

starting hydrogels even when a high proportion of βCD is grafted; and ii) the βCD units grafted to the flexible network can fully develop their complexation ability without steric restrictions. These features provide the hydrogels with huge potential in the development of drug-loaded SCLs and also for other medicated biomedical devices or implants.

Acknowledgements

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Appendix

Figures with essential colour discrimination. Figure 5 in this article may be difficult to interpret in black and white. The full colour image can be found in the on-line version, at doi:10.1016/j.biomaterials.2008.11.016.

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Protein adsorption and miconazol delivery properties of hydrogels functionalized with α-, β- and γ-cyclodextrins.

Enviada.

Protein adsorption and miconazol delivery properties of hydrogels functionalized with α-, β- and γ-cyclodextrins[§]

Jose-Fernando Rosa dos Santos [a], Juan-Jose Torres-Labandeira [a], Nele Matthijs[b], Tom Coenye [b], Angel Concheiro [a], and Carmen Alvarez-Lorenzo [a,*]

[a] Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia. Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain

[b] Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium

*Corresponding author. Tel.: 34 981563100 (14876); fax: 34 981547148; e-mail address: carmen.alvarez.lorenzo@usc.es

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Abstract

pHEMA hydrogels were functionalized with pendant α-, β- and γ-cyclodextrins (CD) with the aim of improving hydrogel biocompatibility and providing them with the capability of CDs to host drug molecules. Functionalization with pendant α-, β- or γ-CDs at two proportion levels did not modify the degree of swelling of the hydrogels but made the water contact angle to slightly decrease. Protein deposition on the hydrogels was notably dependent on the nature of the pendant CDs owing to their different affinity to hydrophobic moieties of proteins. Lysozyme and albumin sorption was hindered by the presence of γ-CD. Functionalization with β-CD also reduced the sorption although less, while α-CD decreased lysozyme deposition but enhanced albumin sorption compared to control pHEMA hydrogels. Loading of the hydrogels with miconazole was carried out by immersion in a drug suspension followed by autoclaving. Functionalization with γ-CD increased twice the affinity of the drug to the network and rendered the highest amount loaded (up to 170 mg/g). Sustained delivery was observed for several days. Some miconazole-loaded hydrogels completely prevented *Candida albicans* biofilm formation as assayed in a microbiological *in vitro* test.

Keywords: *Candida albicans*; contact lens; controlled drug release; hydrogel; fungal biofilm; miconazol.

1. Introduction

The synthesis of biocompatible, comfortable and durable materials to be used for sustained drug delivery is receiving a lot of attention. Synthetic hydrogels occupy an important position among biomaterials owing to their hydrophilicity and ability to absorb water without losing their viscoelastic behavior (*Chien and Lin, 2002*). Poly(2-hydroxyethyl methacrylate), pHEMA, was one of the first components of chemically cross-linked hydrogels and is still widely used due to its biomimetic properties. Furthermore, the mechanical features of pHEMA hydrogels can be easily modulated through copolymerization with comonomers and cross-linking agents of different nature. Thus, pHEMA is still a strong candidate for the development of flexible hydrogel films for topical delivery systems and, particularly, for medicated soft contact lenses (*Horak et al., 2003; Lou et al., 2004; Mabilleau et al., 2006; Tomic et al., 2006; Andrade-Vivero et al., 2007; Satish and Shivakumar, 2007*). Nevertheless, in the absence of specific mechanisms that allow interaction with drug molecules, most hydrogels, including pHEMA hydrogels, show limited loading and poor control of drug release. The aqueous phase of the hydrogel uptakes drug up to reach an equilibrium with the concentration at the surrounding loading solution. Thus if the drug is poorly soluble in water, the concentration achieved inside the hydrogel is also low. Furthermore, once the drug-loaded hydrogel is placed in an aqueous medium, the drug can easily diffuse through the mesh of the network, leading to a fast delivery. Copolymerization of HEMA with monomers capable of establishing specific ionic or hydrophobic interactions with the drug molecules has been widely explored as a way to improve the loading/release behavior (*Ende and Peppas, 1997; Alvarez-Lorenzo et al. 2002; Alvarez-Lorenzo and Concheiro, 2004; Sato et al., 2005*).

Copolymerization with cyclodextrin (CD) derivatives that form inclusion complexes with a given drug has been shown to provide a novel mechanism of drug uptake/retention (*Liu et al., 2004a; Liu et al., 2004b; Kanjickal et al., 2005;*

Siemoneit et al., 2006; Rodriguez-Tenreiro et al., 2007; Dos Santos et al., 2008). The affinity of relatively hydrophobic drugs for the CD cavities enables an efficient loading in the hydrophilic network. Furthermore, since the CDs form part of the backbone of the network, they remain together when the hydrogel enters into contact with physiological fluids and, consequently, drug decomplexation by dilution of the system is minimized. Despite the improvements achieved, copolymerization involves the risk of significant changes in the physicochemical properties of the hydrogel, mainly in the degree of swelling and viscoelastic features (*Dos Santos et al., 2008*). To overcome these drawbacks, we have recently proposed the post-functionalization of preformed hydrogels with pendant cyclodextrins (*Alvarez-Lorenzo et al., 2008; Rosa dos Santos et al., 2009*). The method enables the maintenance of the initial features of the hydrogel since the CDs neither participate nor interfere in the network formation (*Rosa dos Santos et al., 2009).* First, glycidyl methacrylate (GMA) is copolymerized at low proportion with the components of the hydrogel in order to provide the network with binding points for CDs. Once the network is formed, the CD units are attached to the glycidyl moieties through a few of their hydroxyl groups. Functionalization with pendant βCDs was shown to improve diclofenac loading by 1300% and to enhance drug affinity 15-fold (*Rosa dos Santos et al., 2009*).

The aim of the present work was to elucidate the incidence of the functionalization with pendant CD of different nature, namely α-, β- and γ-CD, on the properties of pHEMA hydrogels and, particularly, on loading and release of miconazole. This imidazole antifungal agent alters mycotic cell membrane permeability (*Stevens et al., 1976*), being useful for treatment of *Candida albicans* infections of skin (*Minghetti et al., 1999*), mouth (*Bouckaert et al., 1993; Rindum et al., 1993*) and vagina (*Fothergill et al., 2006*). Sustained delivery of miconazole from hydrogels might be a patient-friendly way of treating the *C. albicans* infections. Furthermore, it can be helpful for preventing infections associated with

the use of medical devices (*Nava-Ortiz et al., 2009*) and thus for reducing the morbidity of nosocomial infections. To test this hypothesis, we evaluated the efficacy of the modified materials to prevent *C. albicans* biofilm formation *in vitro*. Furthermore, the effect of pendant CDs on the pattern of protein adsorption was evaluated. Protein deposition notably determines the compatibility of biomaterials with blood and lachrymal fluid and the adhesion of microorganisms (*Bernacca et al., 1998; Von Eiff et al., 2005; Anderson et al., 2008*). Although surface functionalization with βCD or its derivatives has been reported to change the adsorption pattern of certain biomaterials (*Zhao and Courtney 2007*; *Nava-Ortiz et al., 2009*), the influence of CD variety on the nature and amount of the protein deposited has not been evaluated yet.

2. Materials and methods

2.1. Materials

Ophthalmic grade 2-hydroxyethyl methacrylate (HEMA) was supplied by Merck (Germany). Ethyleneglycol dimethacrylate (EGDMA), glycidyl methacrylate (GMA), albumine bovine serum, lysozyme from hen egg white, sodium dodecylsulfate (SDS) and 2,2'-azo-bis(isobutyronitrile) (AIBN) were from Sigma–Aldrich (Spain). β-Cyclodextrin (βCD) was supplied by Roquette-Laisa (Spain). α-Cyclodextrin (αCD) and γ-Cyclodextrin (γCD) were from Wacker (Germany). Ultrapure water (resistivity > 18.2 MOhm·cm) was obtained by reverse osmosis (MilliQ®, Millipore Spain). Miconazole nitrate was from Fagron Iberica (Spain). All reagents were of analytical grade.

2.2. Phase solubility diagrams

Aliquots of αCD, βCD or γCD aqueous solutions (5 ml) were placed in ampoules containing excess miconazole nitrate (30-35 mg). The systems were autoclaved (Raypa AES-1219, Spain) at 121ºC for 20 min and then shaken at 50 osc/min and

25ºC for 5 days. Finally, they were filtered through 0.22 μm Millipore® cellulose acetate membranes (Teknokroma, Spain) and the concentration of the dissolved drug was measured by UV spectrophotometry (Agilent 8453, Germany) at 272 nm. The apparent stability constant of the drug–CD complexes and the complexation efficiency were calculated from the slope of the plot drug solubility versus CD concentration as follows (*Loftsson et al., 2005*):

$$K_{1:1} = \frac{slope}{S_0(1 - slope)} \quad \text{(Eq. 1)}$$

$$CE = \frac{slope}{(1 - slope)} \quad \text{(Eq. 2)}$$

where S_0 is the drug solubility in absence of cyclodextrins.

2.3. Synthesis of pHEMA hydrogels with pendant cyclodextrins

EGDMA (0.0555 ml, 8 mM) and AIBN (0.0576 g, 10 mM) were dissolved in HEMA (35 ml). The solution was divided into 6 ml portions to which different amounts of GMA (0, 0.082, 0.164, 0.245, 0.327 ml) were added to obtain final concentrations of 0, 100, 200, 300, and 400 mM. After a few minutes of mixing, the solutions were injected into moulds constituted by two glass plates covered internally with a polypropylene sheet and separated by a silicone frame of 0.9 mm thickness. The moulds were then placed in an oven at 50 ºC for 12 h and then heated at 70 ºC for an additional 24 h. After polymerization, each gel sheet was immersed in boiling water for 15 min to remove unreacted monomers and to facilitate the cutting of the disks (10 mm in diameter). The disks were immersed in water for 24 h, then in NaCl 0.9% for 24 h more and finally stored in water. The disks were divided into four groups: a) control group, which did not undergo further treatment; b) αCD-functionalized group, which was immersed in 100 ml of dimethylformamide: 0.5 M NaCl aqueous solution (50:50 v/v) containing 3.0 g NaOH and 1.87 g αCD and kept at 80 ºC for 24 h; c) βCD-functionalized group,

which underwent the treatment described in b) but replacing αCD by 2.2 g βCD; and d) γCD-functionalized group, which underwent the treatment described in b) but replacing αCD by 2.5 g γCD. Then the hydrogels were washed as follows: immersion in water at 80 ºC for 5 min (five cycles), immersion in water at 60 ºC for 24 h (three times), drying at room temperature for 24 h, immersion in ethanol (96%) for 24 h (three times) and drying at room temperature for 48 h. The hydrogel disks were stored at the dried state until used in further tests.

2.4. FTIR

FTIR spectra of the hydrogels were recorded over the range 400–4000 cm^{-1}, in a Bruker IFS 66V FT-IR spectrometer (Germany) using the ATR technique.

2.5. Swelling kinetics

Swelling of dried disks (three replicates) was estimated as the relative weight gain when immersed in water at 25 ºC; the sample being weighed at various times *t* after careful wiping of its surface with a soft tissue:

$$Q_t = 100\ (W_t - W_0)/W_0 \qquad \text{(Eq. 3)}$$

where W_0 is the weight of the dry sample and W_t is the weight at time *t*.

2.6. Surface contact angle measurements

The water contact angle on dried hydrogel disks (in duplicate) was measured in static mode using a Phoenix-300 plus video based optical contact angle measuring device fitted with SurfaceWare software (SEO, Korea). A drop of 10 µl water was deposited on the hydrogel disk and the contact angle was measured during 15 min.

2.7. Cytocompatibility

Cytocompatibility studies were carried out according to the direct contact test of the ISO 10993-5:1999 standard. Modified and non-modified hydrogels were

immersed in USP phosphate buffer pH 7.4 and autoclaved. Then, the disks were added to wells (24 wells plates) containing Balb/3T3 clone A31 cells (200,000 cells per well, 2 ml) in Dulbecco's Modified Eagle's Medium (DMEM) F12 HAM (Sigma-Aldrich, USA) and kept in a humidified incubator at 5% CO_2 and 37ºC. After 24 h, aliquots of medium (100 µl) were taken and mixed with the reaction medium (100 µl) provided with the Cytoxicity Detection KitPLUS (LDH, Roche). Blank (100 µl of medium), negative (50 µl of cells and 50 µl of medium) and positive (50 µl of cells and 50 µl of medium with 5 µl of lysis factor) controls were also prepared. The plates were incubated for 10 min at 15-25 ºC protected from light. Fifty µl of stop solution were added to each well and the absorbance at 490 nm was immediately measured using an ELISA reader. The cytotoxicity was calculated as follows:

$$Cytotoxicity\ (\%) = \frac{Abs_{exp} - Abs_{negative\ control}}{Abs_{positive\ control} - Abs_{negative\ control}} \qquad \text{(Eq. 4)}$$

2.8. Protein interaction

Dried hydrogel disks (six replicates) were soaked in 30 ml pH 7.4 phosphate buffered saline (PBS) medium for 4 h. Then, each disk was placed in 5 ml solution of albumin (1.65 g/L) or lysozyme (0.42 g/L) dissolved in PBS. The changes in protein concentration in the medium were recorded spectrophotometrically at 281 nm for albumin and 279 nm for lysozyme. Protein solutions without hydrogels served as controls for each experiment.

2.9. Miconazole loading and release

Dried hydrogel disks (six replicates) were placed in 60 ml of miconazole nitrate aqueous suspension and autoclaved for 20 min at 121ºC. Immediately after sterilisation, the disks were removed from the medium and dried at 40ºC. To carry out the release tests, the disks were rinsed with water and then immersed in 10-35

ml of 0.3% SDS medium at 25 ºC. The experiments were carried out in triplicate under *sink* conditions. The amount of drug released was measured spectrophotometrically (272 nm) in samples (1 ml) periodically taken and again placed in the same vessel so that the liquid volume was kept constant.

2.10. Antifungal activity

C. albicans SC5314 biofilms were grown on disks made of CD-functionalized hydrogels or silicone disks (which served as control for biofilm formation) in 24-well microtiter plates (TPP, Trasadingen, Switzerland). Silicone sheets were prepared from a medical grade silicone rubber kit (Q7-4735; Dow Corning Corp., Midland, MN, USA) according to the manufacturer's instructions. 13-mm diameter disks were punched from the sheets, subsequently washed in 2% RBS 35 solution (Sigma, St. Louis, MO, USA), rinsed with MilliQ water (Millipore, Billerica, MA, USA) and heat-sterilized. Start cultures were prepared by incubating *C. albicans* cells for 16 h in Sabouraud Dextrose Broth (SDB; Oxoid, Hampshire, UK) at 37°C; cells were subsequently washed three times with and finally resuspended in 1 ml 0.9% (w/v) NaCl. A 0.4 % inoculum was prepared in Yeast Nitrogen Base (YNB; BD, Franklin Lakes, NJ, USA) supplemented with 50 mM glucose (Sigma, St. Louis, MO, USA). One ml of this suspension was added to each well and plates were incubated for 1 h at 37°C. Disks were then washed three times with 1 ml 0.9% (w/v) NaCl to remove non-adherent cells. Disks were placed in new plates, 1 ml diluted YNB (1:5; final glucose concentration: 10 mM) (YNB 0.2x) was added to each well, and the plates were further incubated for 24 h at 37°C.

To enumerate culturable cells in biofilms, plating was used. Disks with biofilms were transferred to 10 mL SDB and sessile cells were removed by three cycles of 30 sec sonication (Branson 3510, 42 kHz, 100 W; Branson Ultrasonics Corp., Danbury, CT, USA) and 30 sec vortex mixing. Using this procedure, all sessile

cells were recovered without compromising their viability and culturability. Serial tenfold dilutions of the resulting cell suspensions were plated on SDA and plates were incubated for 24 h at 37°C, after which colonies were counted. The biofilm experiments were performed on six disks of each material, in at least two independent runs. Independent sample t-tests were carried out using SPSS 15.0 software to determine whether differences were statistically significant ($p<0.05$). Reductions were considered biologically relevant when a statistically significant reduction ($p<0.05$) of at least 1 log unit was observed between the modified miconazole-loaded samples and the modified unloaded samples.

3. Results and Discussion

3.1. Functionalization of pHEMA hydrogels with pendant CDs

Copolymerization of HEMA with GMA rendered hydrogels with glycidyl groups both in the bulk and at the surface of the network that were suitable for reacting with the hydroxyl groups of native CDs under certain conditions (*Rosa dos Santos et al., 2009*). Preliminary studies, using CD alkaline solutions mixed with several polar aprotic solvents, indicated that dimethylformamide is the most suitable one for avoiding chemical damage of the hydrogels and achieving high yield of binding of CDs. Boiling of hydrogels followed by an extensive washing protocol led to the complete extraction of dimethylformamide from the CD cavities and the removal of unreacted chemicals. All hydrogels with pendant CDs showed a smooth surface and a high transparency with a transmittance above 90% at 600 nm.

The ATR infrared spectra of all disks featured the bands characteristic of pHEMA: mainly, hydroxyl groups at 3330-3440 cm^{-1}, C=O amide and ester groups at 1727 cm^{-1}, and ether groups at 1250-1075 cm^{-1}. The bands of glycidyl methacrylate (*Canto and Pessan. 2002*) and cyclodextrin overlapped with those of

pHEMA and it was not possible to quantify them by using the infrared spectra (data not shown).

After immersing the dried disks in water, all of them reached equilibrium within approximately 3 hours (Figure 1). CD-functionalized hydrogels showed a slightly greater degree of swelling (66-72%) than the non-functionalized ones (61%). However the influence of the amount of CD was minor, as the degree of swelling was quite similar for hydrogels prepared with 100 to 400 mM GMA.

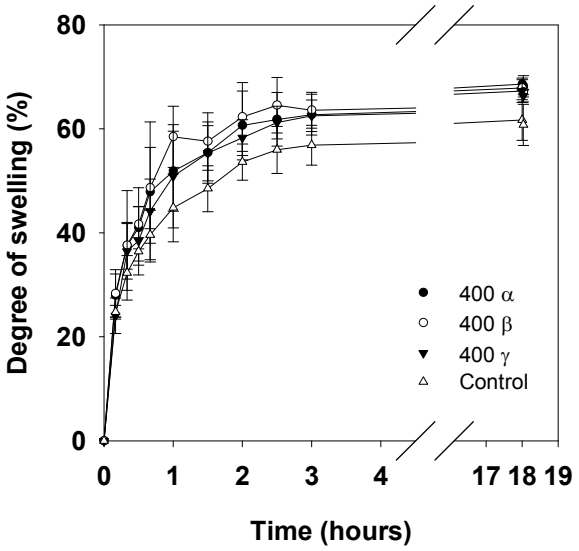

Figure 1. Swelling in water of pHEMA hydrogels (control) and pHEMA-co-GMA (400 mM) hydrogels functionalized with α-, β- or γ-cyclodextrin.

CD-functionalization of hydrogels led to slightly more hydrophilic surfaces, as was evident from decreasing contact angles (Figure 2).

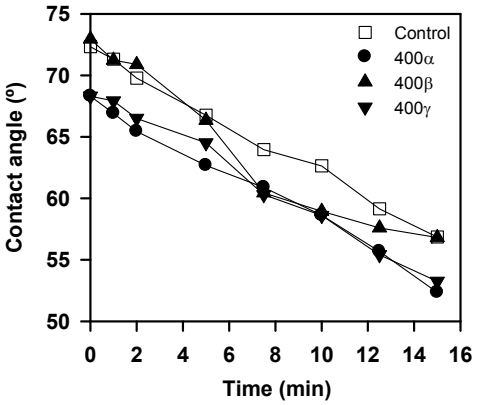

Figure 2. Water contact angle on pHEMA hydrogels (control) and pHEMA-co-GMA (400 mM) hydrogels functionalized with pendant α-, β- or γ-cyclodextrin.

3.2. Protein adsorption and cytocompatibility

An important aspect regarding the biocompatibility of a biomaterial is the protein adsorption pattern at the application site, mainly when intended to enter in contact with blood or lachrymal fluid (e.g. implants or soft contact lenses). Serum albumin concentration in blood is about 25-50 g/L, while the tear fluid undergoes day-cycle variations, changing from open to closed eye between 0.06 to 1.10 g/L. The concentrations of antimicrobial lysozyme (1.6-2.0 g/L) as well as the other reflex tear components (lactoferrin and TSPA) remain practically constant in the tears and are secreted in direct response to neural stimulation (*Sack et al., 1992*). Although proteins and other tear components (e.g. mucin and lipids) may enhance contact lens wettability, it has been reported that once deposited on a lens, the proteins may become denatured and allergenic, contributing to adverse ocular syndromes, e.g. contact lens associated red eye and papillary conjunctivitis (*Hume et al., 2004*). It is known that lysozyme is adsorbed and absorbed to the HEMA-type etafilcon A contact lenses 10 to 20 times more than on silicone-type

hydrogels. On the other hand, albumin adsorbs on the surface of pHEMA but cannot penetrate the polymer network (*Zainuddin et al., 2006*).

In our simplified *in vitro* model, the degree of deposition of proteins on hydrogels with pendant CDs was much lower than on control hydrogels, except when αCD was used (Figure 3). The deposition pattern showed a rapid adsorption followed by a decrease on time for the first 8 hours. Such a pattern has been also reported for other hydrogels (*Zhang et al., 2005*). Lysozyme deposition on control pHEMA hydrogels was in the range of the values previously reported for Etafilcon A (100 to 600 μg per lens) taking into account the greater weight of the present disks (≈ 90 mg) (*Zhang et al., 2005*).

Functionalization with γCD led to a remarkable decrease in the tendency of proteins to adhere to the hydrogel surface. βCD ranked the second in effective prevention of protein deposition. By contrast, αCD at the highest concentration promoted protein adsorption, particularly that of albumin. These findings indicate that, although CDs enhance the hydrophilicity of the hydrogel surface (see Figure 2), the capability of αCD and of βCD to form reversible inclusion complexes with some hydrophobic groups of the amino acids (*Cooper et al., 1996; Yamamoto et al., 2006*) may be responsible for some protein adherence. As expected from the low protein binding constants reported for γCD in solution, the higher the content in this CD on the hydrogel structure, the lower the amount of protein adsorbed.

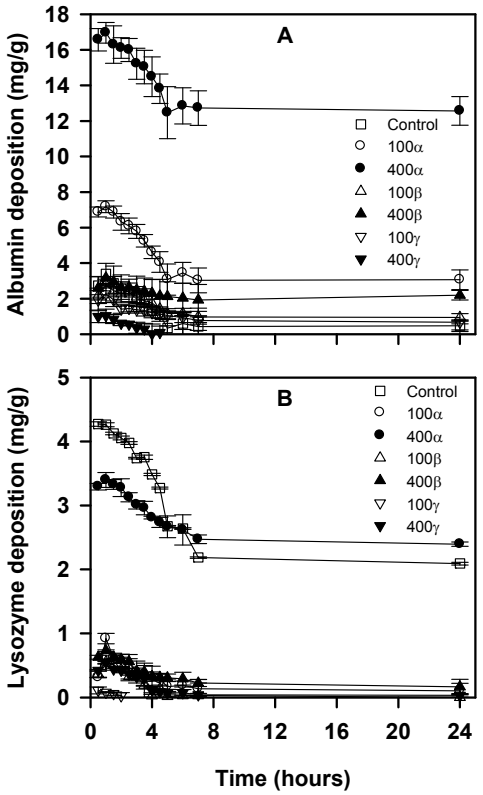

Figure 3. Sorption of albumin and lysozyme by pHEMA hydrogels and pHEMA-co-GMA hydrogels functionalized with pendant α-, β- or γ-cyclodextrin.

Cytocompatibility studies using Balb/3T3 clone A31 cells confirmed the excellent cell compatibility of the CD-functionalized hydrogels, as cell viability > 95% after 24 hours of contact.

3.3. Miconazole-CD affinity constant

The ability of various CDs to form complexes with miconazole has previously been investigated, with the goal to improve its aqueous solubility as well as its therapeutic activity (*Piel et al., 2001; Barillaro et al., 2008*). Recently, Wang and Cai (*2008*) confirmed the inclusion complex formation between β-CD and

miconazole nitrate using IR spectroscopy, differential scanning calorimetry (DSC) and X-ray diffraction. They found that the benzene ring enters into the cavity and that the reaction is spontaneous, reaching the highest yield when the complexes are prepared at acid pH and low temperature. Nevertheless, since the data available in literature about complexation with αCD, βCD, and γCD have been obtained under different experimental conditions, it was not possible to directly compare the miconazole affinity constant for each CD. Thus, prior to the loading studies, the phase solubility diagrams of miconazol in water with each CD were obtained (Figure 4). In the presence of αCD, an A_L type diagram (straight line) of the Higuchi and Connors (*1965*) classification was obtained. The linear plot indicates a straight relationship between drug solubility and CD concentration, revealing a preferential 1:1 inclusion complex. The solubility diagram for βCD was B_s type, which indicates a linear dependence until a certain concentration beyond which the solubility does not increase more. The limited solubility of βCD inclusion complexes explains such a behavior. Finally, A_n type diagram (negative curvature) was obtained with γCD. As pointed out by Higuchi and Connors, negative-curvature diagrams reflect an alteration in the efficiency of the solvent in the presence of high concentrations of the host molecule, leading to a change in the complex formation constant. The affinity constant $K_{1:1}$ was estimated from the initial straight-line portion of the solubility diagrams, and was 436, 596 and 488 M^{-1} for αCD, βCD and γCD, respectively. These values are in agreement with those reported in literature (*Tenjarla et al., 1998*). The complexation efficiency CE (i.e., the concentration ratio between cyclodextrin in a complex and free cyclodextrin (*Loftsson et al., 2005*) was 0.21, 0.29 and 0.24 for αCD, βCD and γCD, respectively. This means that at low CD concentration, the three CD assayed behave quite similar as hosting agents for miconazole. Nevertheless, only αCD and γCD are soluble at high concentrations and are able to enhance drug solubility up to 20-fold and 10-fold, respectively.

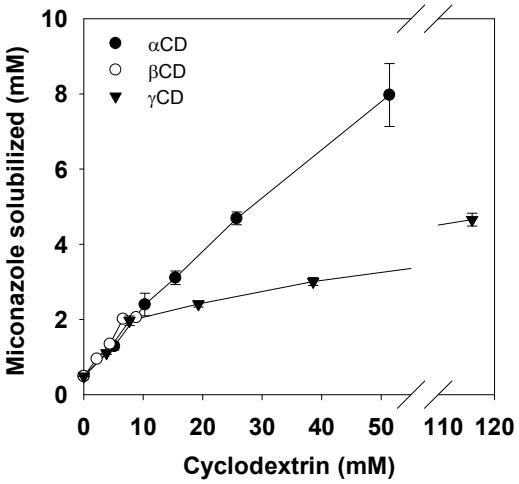

Figure 4. Miconazole phase solubility diagrams in cyclodextrin aqueous solutions.

3.4. Miconazole nitrate loading and release

Loading of the disks was carried out by immersion in a drug suspension followed by autoclaving in order to increase the apparent drug solubility and, thus, the likelihood of complex formation. In such a way, the pendant CDs could fulfill complex formation capability in a short time. Autoclaving did not alter the mechanical properties of the hydrogels. After loading the disks, a release study was performed (Figure 5). The hydrogels were able to sustain the delivery of miconazole during at least one week. It can be observed that despite the fact that the hydrogels swell to a large extent in the first two hours, the amount of miconazole delivered to the aqueous medium during the first day was quite small. Then an almost linear increase in the amount released occurred for 6 days. The profiles did not resemble a diffusion-controlled process but zero-order kinetics after a certain lag time. Miconazole is a relatively hydrophobic drug and may require some time for a complete dissolution even under sink conditions. Nevertheless, a dissolution-controlled release did not explain such a long lag time.

These findings suggest that interactions between the drug and the CD are indeed contributing to the control of the release.

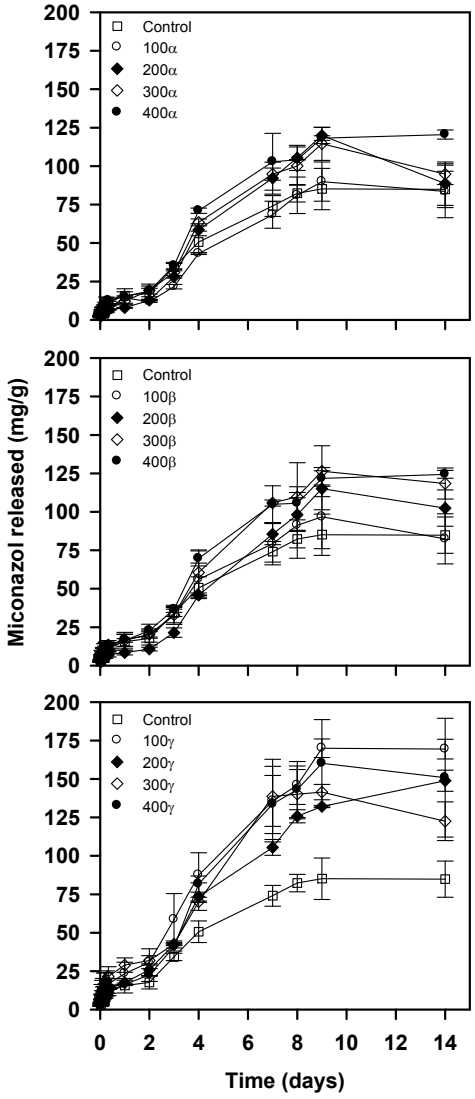

Figure 5. Miconazole release profiles from pHEMA and pHEMA-co-GMA hydrogels functionalized with cyclodextrins.

The amount of drug loaded was significantly higher in the case of pHEMA-co-GMA hydrogels functionalized with γ-CD. The amount loaded by equilibrium

between the aqueous phase of the network and the loading solution, which leads the drug concentration within the hydrogel to be equal to that of the loading solution, can be estimated using the following equation proposed by Kim et al. (*1992*):

$$Loading\ (aqueous\ phase) = (Vs/Wp)xC_0 \qquad \text{(Eq. 5)}$$

where Vs is the volume of water sorbed by hydrogel (mL) and Wp is dried hydrogel weight (g), and C_0 concentration of drug in the loading solution (mg/mL).

Since the loading was carried out by immersion in a miconazole suspension, C_0 is the solubility of miconazole in water, i.e., 0.17 mg/mL *(Tenjarla et al., 1998).* In table 1 the amounts of miconazole loaded by each hydrogel in the aqueous phase are shown. The values are remarkably smaller than the total amount loaded, as estimated from the release tests. This means that the drug establishes hydrophobic interactions with the network and forms inclusion complexes with CD cavities through its aromatic rings. To gain an insight into the role of the interaction of miconazole with the CD units, the partition coefficient, *K*, between the polymer network and the drug loading solution was estimated from the following expression (*Kim et al., 1992*):

$$Loading\ (total) = [(Vs + KVp)/Wp]xC_0 \qquad \text{(Eq. 6)}$$

where *Vp* is the volume of dried polymer (mL) and the other symbols maintain the meaning of the former equation.

The high *K* values obtained (Table 1) indicate that the affinity of miconazole for pHEMA (control) networks is already quite high and that such an affinity can be increased by attaching CDs to the network structure. It should be noted that functionalization with the highest proportion of α- or β-CD leads to relevant increase in the affinity values. The presence of γ-CD contributed at any proportion

evaluated to marked increases in the amount of drug hosted forming inclusion complexes. Roughly, complex formation with γ-CD doubled the amount loaded.

The minimum inhibitory concentration (MIC) of miconazole against *Candida spp.* has been reported to be 0.4-0.8 mg/l (*Piel et al., 1998*). A hydrogel disk of 1 cm^2 can roughly incorporate 5-7 mg of drug, which is enough to prevent the growth of *Candida spp.* in 10 liters of aqueous medium. These results suggest that the hydrogels could be efficient miconazole delivery systems for the local treatment of fungal infections.

Table 1. Amounts of miconazole loaded by the hydrogels (total and in the aqueous phase), network/water partition coefficient *K* and relative values of *K* referred to the control pHEMA hydrogels.

Hydrogel	Total miconazole loaded (mg/g)	Miconazole loaded in the aqueous phase (mg/g)	*K*	Relative *K* values
Control	84.78 (11.78)	0.103 (0.007)	498	1
100 α	83.49 (17.08)	0.120 (0.003)	490	0.98
200 α	88.56 (14.11)	0.120 (0.002)	520	1.04
300 α	94.75 (6.76)	0.117 (0.002)	557	1.12
400 α	120.50 (2.94)	0.116 (0.004)	708	1.42
100 β	82.40 (16.31)	0.119 (0.004)	484	0.97
200 β	102.40 (11.76)	0.122 (0.002)	602	1.21
300 β	118.35 (10.12)	0.119 (0.006)	696	1.40
400 β	124.32 (2.56)	0.114 (0.001)	731	1.47
100 γ	169.36 (6.41)	0.117 (0.004)	996	2.00
200 γ	148.78 (6.83)	0.119 (0.001)	874	1.75
300 γ	140.06 (12.64)	0.117 (0.005)	823	1.65
400 γ	150.76 (18.66)	0.113 (0.002)	886	1.78

3.5. Antifungal activity

Ideally, miconazole-loaded hydrogels should be able both to deal with already established fungal infections and to prevent the growth of microorganisms on their surface. In such a way they could be used for therapy or for prophylaxis depending on their intended use as drug delivery systems or as components of biomedical devices. Microorganisms tend to adhere to the surface of living tissues and of synthetic materials forming biofilms that protect them from innate defense mechanisms, promoting their persistence and invasion into host tissues (*Dongari-Bagtzoglou, 2008*). Fungi inside the biofilm (sessile cells) show marked genotypic and phenotypic differences when compared to their planktonic (free-living) counterparts, including increased antimicrobial resistance (*Mah and O'Toole, 2001; Schierholz and Beuth, 2001*). The capability of the drug-loaded hydrogels to prevent *C. albicans* biofilm formation was the subject of the microbiological test.

The disks were immersed for 1 hour in *C. albicans* inoculum and then the biofilms were allowed to form on each disk for 24 h at 37°C. Non-loaded hydrogels showed biofilms with a high number of sessile *C. albicans* cells, comparable to the number of cells recovered from medical-grade silicone disks used as controls (1.7 and 4.7 x 10^6 CFU/disk, respectively) (Figure 6). In contrast, the hydrogels that loaded the highest amounts of miconazole exhibited a statistically significant decrease in biofilm formation, ranging from 99.98772% (400 β-CD) to 99.99995% (100 γ-CD) On most γ-CD functionalized hydrogels no growth at all was observed indicating that *C. albicans* biofilm formation was completely inhibited.

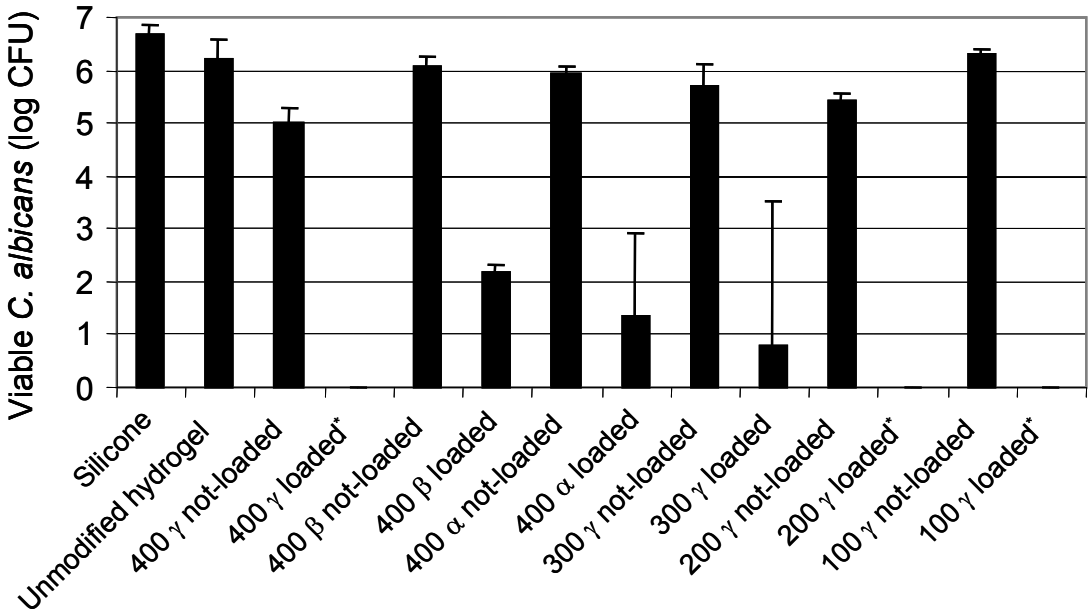

Figure 6. Number of sessile *Candida albicans* cells (log CFU per disk, average and standard deviation; n=3) recovered from various disks containing different hydrogels. *No culturable cells were recovered from these disks.

4. Conclusion

Optically transparent, flexible and cytocompatible hydrogels can be prepared by grafting of natural cyclodextrins to preformed pHEMA-co-GMA hydrogels taking advantage of the reactivity of GMA for covalent bonding with hydroxyl groups of cyclodextrins. Grafting of βCD or γCD diminishes the trend of proteins to deposit on the hydrogels, which make a good compatibility with blood and lachrymal fluid foreseeable. On the other hand, the greater affinity of αCD for hydrophobic moieties of seroalbumin and lysozyme enhances protein adsorption on hydrogels with pendant αCD. The pendant cyclodextrins improves the capability of the

hydrogels to load so hydrophobic drugs as miconazole. Particularly, γCD doubles the amount of drug uptaken by the hydrogels endowing them with the feature of being able to completely prevent *Candida albicans* biofilm formation.

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4. Resultados y Discusión

4. RESULTADOS Y DISCUSIÓN

4.1. Hidrogeles sintetizados por copolimerización de monómeros de ciclodextrina con monómeros acrílicos (pHEMA-co-βCD)

4.1.1. Síntesis del monómero de βCD

Como primer paso para preparar hidrogeles acrílicos de CD, se sintetizó un derivado de βCD que se copolimerizó con hidroxietil metacrilato (HEMA). El HEMA es un monómero acrílico de uso habitual en la preparación de hidrogeles para aplicaciones biomédicas. Se utilizó el método desarrollado por Saito y Yamaguchi (*Saito y Yamaguchi, 2003*) que conduce a la obtención de 2,3-di-*O*-metacrilato-6-metacrilato-βCD (Fig. 4.1), que es miscible con HEMA. Primeramente se desecó la βCD y se disolvió en piridina. A continuación, se añadió metacrilato anhidro, se agitó durante 2 horas a temperatura ambiente y se calentó a 50ºC durante 5 horas. La concentración de metacrilato anhidro se fijó en 2.2 M para obtener relaciones molares metacrilato:βCD 20 y 40. Finalmente, la mezcla de reacción se vertió sobre agua fría (300 ml) y el sistema se mantuvo a 4ºC durante 12 h. El precipitado se separó por filtración, se disolvió en etanol (20 ml) y se precipitó por adición de agua fría (Fig. 4.2).

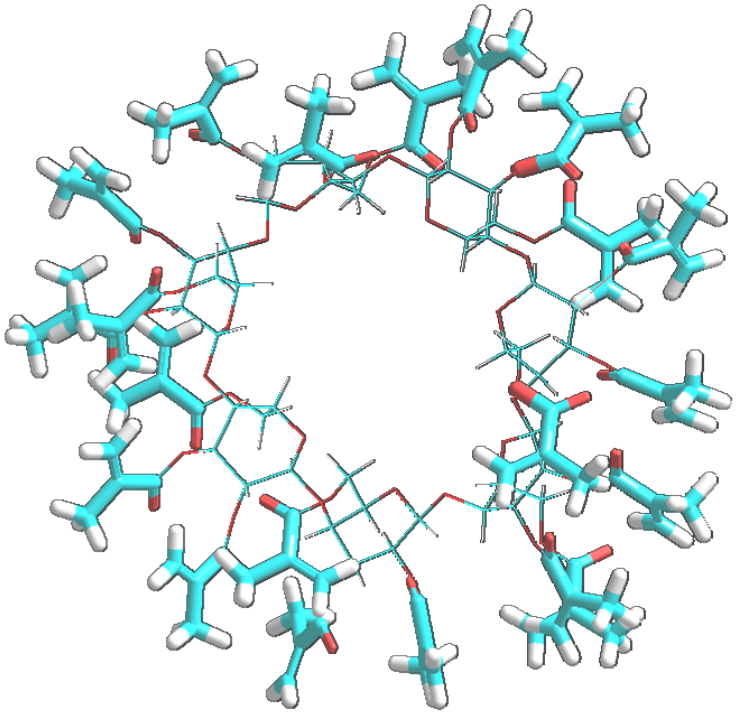

Fig. 4.1. Estructura del 2,3-di-O-metacrilato-6-metacrilato-βCD. Los grupos metacrilato se representan con trazos más gruesos.

Fig. 4.2. Etapas del proceso de síntesis del monómero 2,3-di-O-metacrilato-6-metacrilato-βCD.

La formación del monómero de CD se puso de manifiesto por la presencia en el espectro de FT-IR de una banda característica del enlace éster a 1720cm^{-1} y por un incremento en la absorbancia a 1638cm^{-1}, que corresponde a una banda específica del enlace C=C.

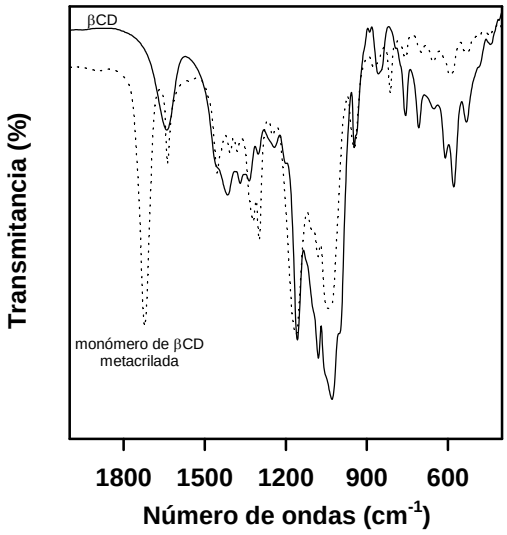

Fig. 4.3. Espectros FT-IR de la βCD y del monómero 2,3-di-O-metacrilato-6-metacrilato-βCD.

Los resultados de los análisis de ^{1}H NMR permitieron concluir que todos los grupos hidroxilo del monómero de βCD se encuentran sustituidos por grupos metacrilato, con las dos relaciones metacrilato:βCD 1:20 y 1:40 utilizadas en la síntesis. El rendimiento del proceso se estimó, asumiendo que el peso molecular del monómero metacrilado es 2311Da, en 85 ± 5%. Los resultados de los análisis de calorimetría diferencial de barrido (DSC) fueron similares a los que se obtuvieron con la βCD, mostrando únicamente el pico característico de la

eliminación de agua entre 100-110ºC. La temperatura de descomposición se situó por encima de 250ºC.

4.1.2. Síntesis de hidrogeles pHEMA-co-βCD

El monómero HEMA, que se incorporó como componente mayoritario, conduce a la formación de entramados de elevada estabilidad química y térmica, que presentan propiedades mecánicas muy versátiles y una excelente biocompatibilidad (*Horak y col., 2003; Lou y col., 2004; Mabilleau y col., 2006; Tomic y col., 2006; Andrade-Vivero y col., 2007; Satish y Shivakumar, 2007*). El agente reticulante etilenglicol dimetacrilato (EGDMA) es un diester formado por condensación de dos unidades de ácido metacrílico y una de etilenglicol, que se utiliza de manera habitual para obtener estructuras flexibles con gran capacidad de hinchamiento en medio acuoso.

El monómero metacrílico de βCD (0.25g a 2g) se mezcló con EGDMA (8 ó 80mM) y HEMA (6ml), se incorporó el iniciador 2,2´-azo-bisisobutironitrilo (AIBN) y se llevó la mezcla a moldes ortoédricos, que se mantuvieron a 50ºC durante 12 horas y a 70ºC 24 horas más (Fig. 4.4). Los hidrogeles se sumergieron en agua en ebullición para eliminar los restos de monómeros no reaccionantes y, al cabo de 15 minutos, se extrajeron y se cortaron en forma de discos. Estos discos se mantuvieron en NaCl (10mM) durante 3 días y en agua durante otros 3 días más. Finalmente se secaron a 50ºC.

Fig. 4.4. Etapas del procedimiento de síntesis de los hidrogeles pHEMA-co-βCD.

4.1.3. Caracterización de los hidrogeles pHEMA-co-βCD

Se obtuvieron discos homogéneos y transparentes, con valores de transmitancia a 600 nm superiores al 90%. Los espectros de FT-IR mostraron las bandas características del pHEMA que corresponden a los grupos hidroxilo (3330-3440 cm^{-1}), carbonilo de los grupos amida y de los grupos éster (1724cm^{-1}), y éter (1250-1075cm^{-1}), y el hombro a 1022 cm^{-1} que es característico de las CDs (Fig. 4.5).

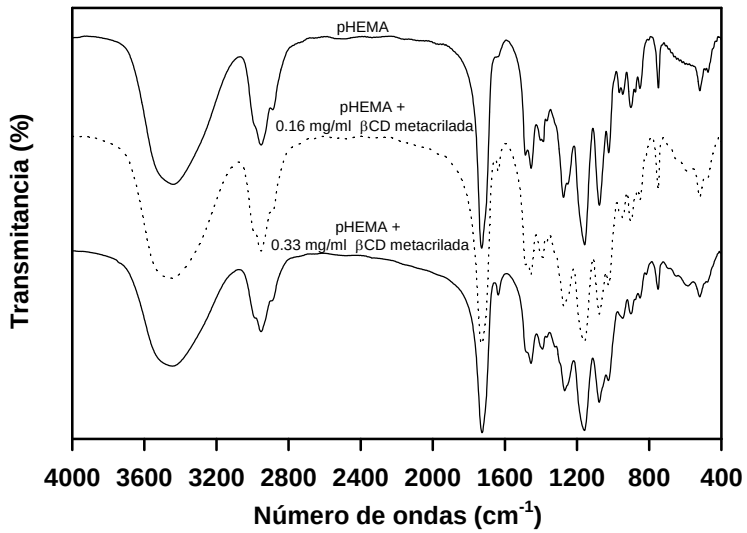

Fig. 4.5. Espectros de FT-IR de los hidrogeles de pHEMA y de pHEMA-co-βCD.

El grado de conversión de los dobles enlaces estimado a través de la relación de absorbancias de la banda característica de C=C antes y después de la polimerización, utilizando la expresión:

$$GC = \left(1 - \frac{\frac{A^{hidrogel}_{1637\ cm^{-1}}}{A^{hidrogel}_{1724\ cm^{-1}}}}{\frac{A^{monómeros}_{1637\ cm^{-1}}}{A^{monómeros}_{1724\ cm^{-1}}}} \right) * 100\%$$

resultó ser del 74%. Sin embargo, el hecho de que las CDs presenten una banda de absorción a 1637cm^{-1}, que se solapa con la de los dobles enlaces, sugiere que el grado de conversión real de los dobles enlaces supera en realidad este valor.

Con la proporción más alta de monómero de βCD se obtuvieron hidrogeles con una elevada temperatura de transición vítrea, T_g, que presentaron una elevada fragilidad y tendencia a astillarse una vez desecados (Tabla 4.1). Los hidrogeles control de pHEMA mostraron una T_g de 110ºC; un valor concordante al publicado previamente para estos materiales (*Andrade-Vivero y col., 2007*).

Metacrilato-βCD (g/ml)	**EGDMA** (mM)	**T_g** (ºC)
-	80	110
0.042	80	119
0.083	80	149
0.125	80	152
0.167	80	>300ºC
0.250	80	>300ºC
0.333	80	>300ºC

Tabla. 4.1. Temperatura de transición vítrea de los hidrogeles de pHEMA-co-βCD.

La dependencia del módulo elástico o de almacenamiento, G´, y del módulo viscoso o de pérdida, G´´, respecto de la temperatura aporta información muy útil acerca del modo en que los cambios térmicos pueden afectar a las propiedades estructurales de los entramados. Los resultados obtenidos en este estudio indican que cuanto mayor es el contenido en βCD, menor es la sensibilidad del entramado a los cambios de temperatura (Fig. 4.6A). Los hidrogeles secos preparados con proporciones intermedias de metacrilato-βCD se mantienen en estado vítreo al menos hasta los 200ºC, mostrando valores elevados de G´ y G´´.

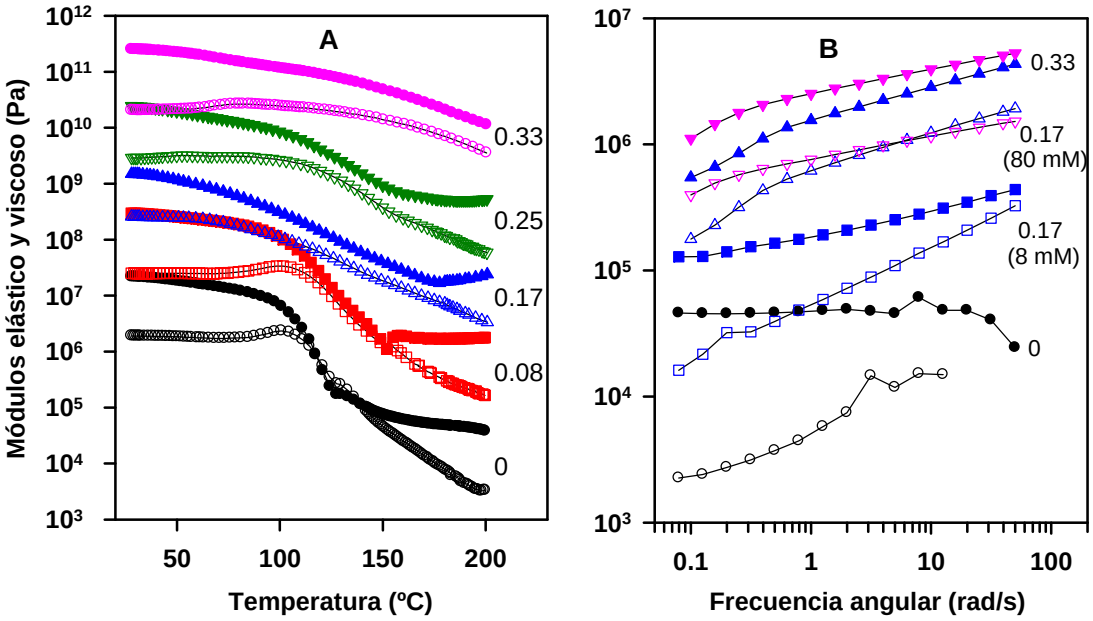

Fig. 4.6. Propiedades reológicas de los hidrogeles de pHEMA-co-βCD, elaborados con distintas cantidades de metacrilato-βCD (g/ml de solución monomérica): (A) hidrogeles secos y (B) hidrogeles hidratados. Los valores correspondientes a los hidrogeles con 0.083, 0.167, 0.250 y 0.333 g/ml se desplazaron en el gráfico A unos 10, 100, 1000 y 10000 Pa, respectivamente, para facilitar la visualización.

Los numerosos grupos reactivos del monómero metacrilato-βCD (Fig. 4.1) hacen que actúe como agente reticulante, por lo que su presencia contribuye a incrementar la rigidez del entramado. En la Tabla 4.2 se muestra la influencia de de la concentración de metacrilato-βCD en la mezcla de reacción sobre el grado de reticulación del entramado. Para estimar el número de monómeros entre puntos de reticulación se utilizó la expresión:

$$N = \frac{[HEMA]}{2[EGDMA] + 21[metacrilato - \beta\mathrm{CD}]}$$

Metacrilato-βCD g/ml	**0**	**0.042**	**0.083**	**0.125**	**0.167**	**0.250**	**0.333**
EGDMA = 80mM	48	14.3	8.4	5.9	4.6	3.2	2.4
EGDMA = 8mM	480	19.5	9.9	6.7	5.0	3.3	2.5

Tabla. 4.2. Unidades de HEMA entre puntos de reticulación.

En su conjunto, los resultados obtenidos en este apartado del estudio prueban que el monómero metacrilato-βCD causa una reducción marcada de la longitud de los segmentos poliméricos entre puntos de entrecruzamiento, lo que limita la movilidad de las cadenas. Este efecto se pone de manifiesto con incrementos marcados en el valor de la T_g.

La efectividad como agente reticulante del metacrilato-βCD también se puso de manifiesto a través del comportamiento viscoelástico de los hidrogeles hidratados. Los valores de G´ y G´´ -casi 100 veces inferiores a los observados para los hidrogeles secos debido al efecto plastificante del agua- resultaron ser independientes de la frecuencia angular, como ocurre con los entramados bien estructurados. Para una proporción constante de EGDMA, los valores de G´ y G´´ van aumentando a medida que se incrementa la proporción de metacrilato-βCD (Fig. 4.6B). No obstante, en todos los casos los valores de G´ y G´´ se situaron en un intervalo que asegura la flexibilidad y la resistencia mecánica que requieren los materiales que se utilizan habitualmente como componentes de lentes de contacto, implantes o sistemas de liberación de medicamentos (*Refojo y Leong, 1981*).

La capacidad de los hidrogeles para incorporar agua se redujo a medida que se incrementó la proporción de metacrilato de βCD (Fig. 4.7; Tabla 4.3). El carácter relativamente hidrofóbico de este monómero y su efectividad como agente reticulante explican este efecto.

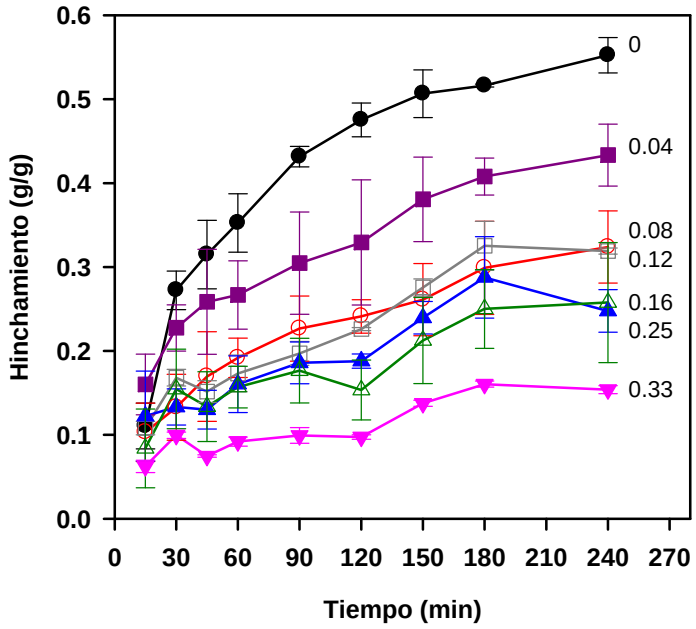

Fig. 4.7. Perfiles de hinchamiento de los hidrogeles de pHEMA-co-βCD.

Desde el punto de vista de la funcionalidad como componentes de dispositivos biomédicos y sistemas de liberación de medicamentos, es importante conocer el estado en el que se encuentra el agua que incorporan los hidrogeles a su estructura. La proporción de agua libre tiene una influencia directa sobre las restricciones al movimiento de solutos a través del entramado. El contenido en agua libre de los hidrogeles de pHEMA-co-βCD se determinó mediante el análisis del pico de cristalización/fusión registrado por calorimetría diferencial de barrido

(Tabla 4.3). La proporción de agua libre se redujo progresivamente al aumentar la proporción de metacrilato-βCD, hasta el punto de que en los hidrogeles con la proporción más alta de metacrilato-βCD no se pudo detectar. Por lo tanto, en estos entramados la difusión de los fármacos se podría ver dificultada.

Metacrilato-βCD (g/ml)	**Hinchamiento** (%)	**Agua libre** (% sobre agua total)
0	60.4 (0.7)	15.5
0.042	53.3 (1.6)	8.0
0.083	48.1 (2.1)	6.1
0.125	42.0 (1.1)	6.3
0.167	38.0 (1.3)	6.1
0.250	32.2 (0.8)	1.3
0.333	29.5 (0.6)	No detectable

Tabla. 4.3. Grado de hinchamiento y porcentaje de agua libre de los hidrogeles pHEMA-co-βCD una vez alcanzado el equilibrio.

4.1.4. Contenido en ciclodextrinas con capacidad de formar complejos de inclusión

Para evaluar la influencia de la estructura del entramado sobre la accesibilidad de las cavidades de las ciclodextrinas a moléculas pequeñas y, en consecuencia, sobre la capacidad de formación de complejos de inclusión, se utilizó como marcador el ácido 3-metilbenzoico (3-MBA) (Fig. 4.8). Esta molécula cuenta con una reconocida afinidad por la βCD ($K=1.3\times10^7$ M^{-1}) (*Fundueanu y col., 2003*), que determina que en presencia de esta ciclodextrina, la concentración de 3-MBA libre en el medio sea muy reducida

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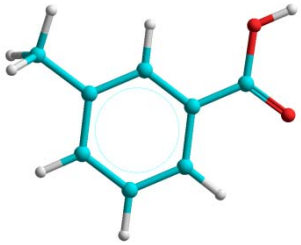

Fig. 4.8. Ácido 3-metilbenzoico.

Para valorar el contenido en ciclodextrina con capacidad de formar complejo de inclusión, se sumergieron los hidrogeles en 10ml de una disolución de 3-MBA (0.5 mg/ml) y el sistema se mantuvo en agitación durante 48 horas. La cantidad de 3-MBA retenida por la βCD en los hidrogeles se determinó por espectrofotometría de UV (Agilent 8453, Alemania) a 281nm. Para determinar la cantidad de 3-MBA alojada en la fase acuosa se utilizó un hidrogel control sin βCD. La cuantificación de 3-MBA que participa en la formación de complejos de inclusión se hizo por diferencia entre la cantidad total cargada y la cantidad

alojada en la fase acuosa. Como se muestra en la tabla. 4.4, los hidrogeles sin metacrilato-βCD captan cantidades de 3-MBA sensiblemente más bajas. Por otra parte la relación molar marcador:monómero de βCD se reduce a medida que aumenta la proporción de βCD. Para hidrogeles sintetizados con 0.042-0.125g/ml de metacrilato-βCD, la carga de moléculas de 3-MBA por molécula de βCD es superior a la unidad, lo que está en consonancia con la tendencia del 3-MBA a formar dímeros o incluso agregados de orden superior en medios apolares (*Rassing y col., 1967*). En cambio, en los hidrogeles preparados con 0.167g/ml o proporciones más altas de metacrilato-βCD, la cantidad de 3-MBA fue inferior a la requerida para que todas las unidades de βCD se encuentren en forma de complejo con, al menos, una molécula de 3-MBA. Este comportamiento se explica por el reducido tamaño de malla de estos hidrogeles, debido a su alto grado de reticulación y a la consiguiente dificultad con la que el 3-MBA difunde en el interior del entramado. El impedimento estérico generado por la proximidad entre unidades de βCD también puede dificultar la complejación. No obstante, incluso en los hidrogeles con mayor contenido en βCD, se constató que por lo menos un 42% de las cavidades están disponibles para formar complejos.

[metacrilato-βCD] g/ml	EGDMA (mM)	Carga de 3-MBA		
		Total (mg/g de gel seco)	Cargado en CD (mg/g de gel seco)	Mol/mol CD
0	80	6.18 (1.10)	0	0
0.042	80	11.38 (0.41)	5.2	2.46 (0.18)
0.083	80	12.18 (0.11)	6	1.52 (0.03)
0.125	80	11.68 (0.98)	5.5	1.01 (0.16)
0.167	80	13.07 (0.02)	6.89	0.88 (0.01)
0.250	80	13.46 (1.45)	7.28	0.76 (0.02)
0.333	80	10.52 (0.63)	4.34	0.42 (0.04)
0	8	10.43 (0.11)	0	0
0.083	8	14.17 (0.87)	3.74	1.05 (0.12)
0.167	8	14.41 (0.91)	3.98	0.68 (0.02)
0.250	8	15.52 (0.85)	5.09	0.63 (0.02)

Tabla. 4.4. Cantidades de 3-MBA incorporadas a los hidrogeles de pHEMA-co-βCD sintetizados con distintas proporciones de agente reticulante EGDMA. Valores medios y, entre paréntesis, desviación estándar.

4.1.5. Citocompatibilidad

Para evaluar la biocompatibilidad de los hidrogeles, se acudió a un ensayo de citocompatibildad utilizando una línea celular de macrófagos (RAW 264.7) sensible a los doble enlaces reactivos que pudieran permanecer en el entramado como consecuencia de una incompleta polimerización y a los monómeros que se vayan liberando del hidrogel (*Lin y col., 2007*). Los macrófagos se pueden encontrar en cualquier zona del organismo, participando de forma activa en los procesos inflamatorios y en la presentación de antígenos durante los procesos

infecciosos. Cuentan con capacidad para producir mediadores primarios del proceso inflamatorio (citoquinas, interleuquinas-1β y factor de necrosis tumoral-α (TNF-α)) y actúan como amplificadores de señales para activar otras células.

El ensayo de citocompatibilidad se llevó a cabo en placas de 12 pocillos en las que se ensayaron, por triplicado, discos de hidrogel previamente autoclavados en tampón fosfato (pH 7.4). Se sembraron 5×10^5 células RAW en 2ml de medio DMEM suplementado con suero fetal bovino y gentamicina, que se incubaron a 37ºC y 5% CO_2 durante 10 días. Al cabo de 1, 5 y 10 días se extrajeron alícuotas del medio y se congelaron inmediatamente a -20ºC para su posterior análisis.

La viabilidad celular se analizó por microscopia confocal. Los discos se retiraron de los pocillos después de 5 días de cultivo, se lavaron con medio DMEM y se tiñeron las células con calceina (14 mg/ml) y ioduro de propidio (50 mg/ml). La calceina tiñe células viables, que adquieren coloración verde, mientras que el ioduro de propidio tiñe células muertas, que adquieren coloración roja. Después de la incubación, las muestras se lavaron con DMEM para eliminar el exceso de colorante y se observaron en el microscopio de confocal (Fig. 4.9). Tras cinco días de contacto de los discos con las células, la viabilidad celular siguió siendo excelente. Es importante destacar que si se hubieran cedido monómeros tóxicos, aun en muy pequeñas cantidades, sus efectos sobre la viabilidad celular se habrían puesto de manifiesto dado el reducido volumen de medio en el que se llevaron a cabo los ensayos.

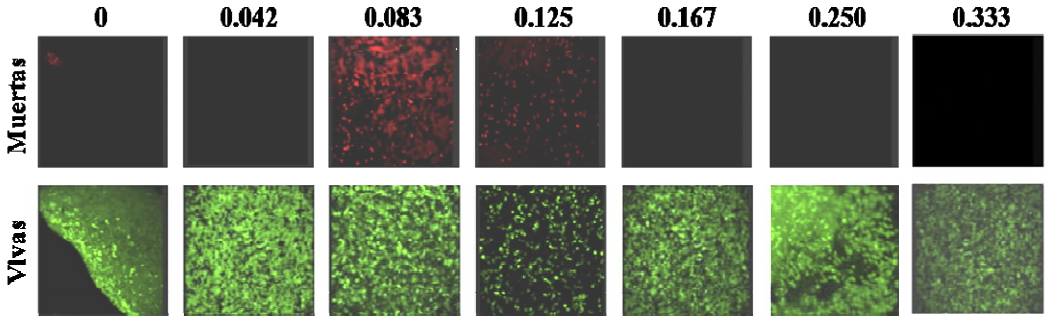

Fig. 4.9. Viabilidad de células RAW 264.7 al cabo de cinco días de cultivo en presencia de discos de hidrogel sintetizados con distintas proporciones de metacrilato-βCD.

También se monitorizó la producción de los marcadores TNF-α y IL-1α. El TNF-α o factor de necrosis tumoral es una citoquina polipeptídica que producen los macrófagos para modular la respuesta inmune, que actúa como agente pirogénico e induce la muerte celular. En la cascada de factores que desencadenan la respuesta inmune, el TNF-α es uno de los primeros que se segrega al medio, por lo que resulta muy útil como marcador en la fase aguda de procesos inflamatorios o de muerte celular. Por su parte, la IL-1α es una proteína que se libera como consecuencia de la producción, aun en muy bajas cantidades, de TNF-α. Al cabo de 1, 5 y 10 días no se detectó la presencia de TNF-α o de IL-1α (límites de detección del kit de ELISA 31.3pg/ml para TNF-α y 7.8pg/ml para IL-1α). Por lo tanto, ni el entramado del hidrogel ni los monómeros residuales que hipotéticamente se pudieran desprender de él, son tóxicos ni inducen respuesta inflamatoria.

4.1.6. Carga y cesión de acetazolamida e hidrocortisona

La cantidad de fármaco que carga un hidrogel depende de la afinidad del fármaco por el entramado y de la concentración de la disolución de carga (*Kim y col., 1992*). Para evaluar la capacidad de incorporación de fármaco de los hidrogeles de pHEMA-co-βCD, se eligieron la acetazolamida, de carácter hidrofílico, y la hidrocortisona, de marcado carácter hidrofóbico, para los que ya se puso de manifiesto su capacidad para formar complejos bajo ciertas condiciones (*Loftsson y col., 2005a*).

La acetazolamida (fig. 4.10) es una sulfonamida no bacteriostática que actúa como potente inhibidor de la anhidrasa carbónica, lo que la hace muy efectiva en el control de la secreción de fluídos y muy útil en el tratamiento de algunos tipos de glaucoma (*Kaur y Aggarwal, 2005*).

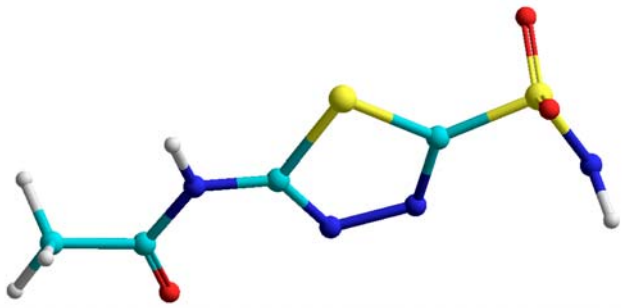

Fig. 4.10. Conformación espacial de la acetazolamida.

La hidrocortisona o cortisol (fig. 4.11) se utiliza en el tratamiento de los procesos alérgicos e inflamatorios que afectan a la conjuntiva, la córnea y el segmento anterior del ojo.

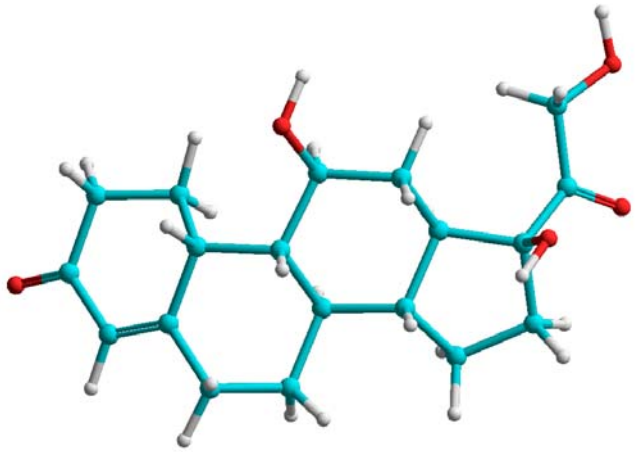

Fig. 4.11. Conformación espacial de la hidrocortisona.

Para determinar las constantes de afinidad de los fármacos por la βCD, se construyeron y se analizaron los diagramas de solubilidad. Los valores de constante de afinidad obtenidos, 58.6 M^{-1} y 988.4 M^{-1} para la acetazolamida y la hidrocortisona respectivamente, pusieron de manifiesto que la tendencia de la hidrocortisona a formar complejos es muy superior a la de la acetazolamida.

La incorporación de los fármacos a los hidrogeles se llevó a cabo por dos procedimientos: a) inmersión en la disolución de fármaco (5 ml de acetazolamida 100 mgL^{-1} ó 10 ml de hidrocortisona 100 mgL^{-1}) durante 4 días a temperatura ambiente; y b) inmersión en la disolución del fármaco,seguido de autoclavado a 121º 20min y almacenamiento en contacto con la disolución de carga durante 4 días en las condiciones indicadas en a). La carga se cuantificó por diferencia entre

las concentraciones inicial y final de fármaco (Tabla 4.5). La cantidad de hidrocortisona incorporada se va reduciendo a medida que aumenta la concentración del metacrilato-βCD en el entramado. Este efecto es especialmente manifiesto con la proporción más elevada de EGDMA (80mM). Con la acetazolamida se observó un comportamiento diferente, alcanzándose la carga máxima con los hidrogeles preparados con proporciones intermedias de metacrilato-βCD (0.125g/ml y 0.167g/ml).

Metacrilato-βCD (g/ml)	**EGDMA** (mM)	**Hidrocortisona** (mg/g gel seco)		**Acetazolamida** (mg/g gel seco)	
		No autoclavado	Autoclavado	No autoclavado	Autoclavado
0	80	1.72 (0.12)	1.81 (0.17)	0.387 (0.008)	0.339 (0.007)
0.042	80	1.57 (0.12)	1.78 (0.07)	0.503 (0.004)	0.447 (0.002)
0.083	80	1.60 (0.17)	1.70 (0.02)	0.643 (0.047)	0.615 (0.072)
0.125	80	1.27 (0.07)	1.29 (0.11)	0.740 (0.087)	0.610 (0.009)
0.167	80	1.05 (0.11)	1.23 (0.03)	0.694 (0.036)	0.597 (0.024)
0.250	80	0.64 (0.09)	0.78 (1.11)	0.591 (0.038)	0.477 (0.064)
0.333	80	0.62 (0.04)	0.76 (0.19)	0.530 (0.064)	0.430 (0.012)
0	8	2.17 (0.37)	2.19 (0.26)	0.304 (0.049)	0.492 (0.018)
0.083	8	2.65 (0.37)	2.44 (0.22)	0.521 (0.065)	0.730 (0.040)
0.167	8	1.54 (0.04)	1.66 (0.38)	0.570 (0.063)	0.934 (0.039)
0.250	8	1.42 (0.17)	1.31 (0.02)	0.538 (0.076)	0.984 (0.048)

Tabla. 4.5. Cantidad de hidrocortisona y acetazolamida incorporada por los hidrogeles de pHEMA.

La hidrocortisona, al igual que otras moléculas de naturaleza esteroídica, interacciona con el entramado de los hidrogeles de pHEMA de forma inespecífica a través de puentes de hidrogeno y de interacciones hidrofóbicas (*Sreenivasan, 1999*). La posibilidad de que se establezcan interacciones de este tipo se reduce a medida que se incrementa la concentración en metacrilato-βCD. La presencia de βCD ofrece la posibilidad de que se formen complejos, pero reduce el tamaño de malla del entramado y el volumen de fase acuosa del hidrogel. El predominio de la influencia del segundo factor explica la correlación directa entre el grado de hinchamiento y la cantidad de fármaco cargada (Fig. 4.12).

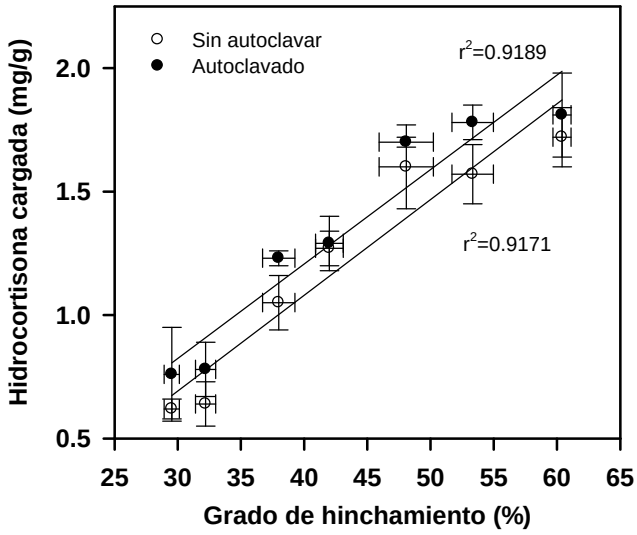

Fig. 4.12. Correlación entre el grado de hinchamiento y la carga de hidrocortisona en hidrogeles de pHEMA-co-βCD con 80mM EGDMA.

La molécula de acetazolamida es mucho más pequeña que la de hidrocortisona (8.96×3.43 *vs.* 13.61×6.06 Å) y, consecuentemente, el tamaño de malla del hidrogel no desempeña un papel tan determinante en el proceso de

carga. Además, la afinidad relativamente baja de la acetazolamida por el pHEMA determina que el proceso de complejación asuma un papel más destacado en el proceso de carga. De hecho los hidrogeles con una proporción intermedia de βCD cargaron el doble que los hidrogeles sin βCD (Tabla. 4.6; Fig. 4.13). No obstante, incluso en estas condiciones el grado de ocupación de las βCDs fue inferior al 10%. Al igual que para la hidrocortisona, la disminución de la capacidad de carga que presentan los hidrogeles preparados con las proporciones más altas de monómero de βCD se puede atribuir a la reducción del tamaño de malla y a impedimientos estéricos.

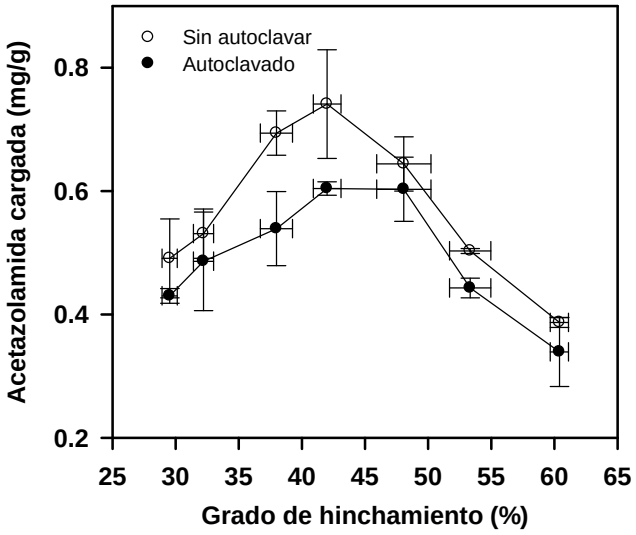

Fig. 4.13. Relación entre el grado de hinchamiento y la carga de acetazolamida en hidrogeles de pHEMA-co-βCD con 80mM EGDMA.

La aplicación de autoclavado incrementó la carga del hidrogel con acetazolamida, lo que prueba que con este fármaco el tratamiento térmico facilita

la movilidad dentro del entramado y promueve su complejación con la CD (*Loftsson y col., 2005a*). Con la hidrocortisona no se observaron mejoras significativas, lo que confirma que las interacciones con la βCD desempeñan un papel secundario en el proceso de carga en comparación con las interacciones inespecificas con el pHEMA.

Si se asume que un fármaco interacciona exclusivamente con el entramado de pHEMA, es previsible que el perfil de cesión sea independiente de la proporción de metacrilato-βCD presente en el entramado, salvo si los cambios en el tamaño de malla del hidrogel afectan significativamente a la difusión de las moléculas de fármaco. Por el contrario, si el fármaco forma complejo con la βCD, la cesión vendrá determinada por la difusión del fármaco libre -que se encuentra disuelto en la fase acuosa o interaccionando inespecíficamente con pHEMA- y por la constante de afinidad del fármaco por la βCD. En este caso, un incremento en la proporción de βCD se reflejará en la ralentización del proceso de cesión.

Los hidrogeles cargados con hidrocortisona mostraron perfiles de cesión sostenida durante un mínimo de 10 días, muy similares entre sí independientemente de la proporción de βCD y de que hubieran sido autoclavados o no durante el proceso de incorporación del fármaco (Fig. 4.14).

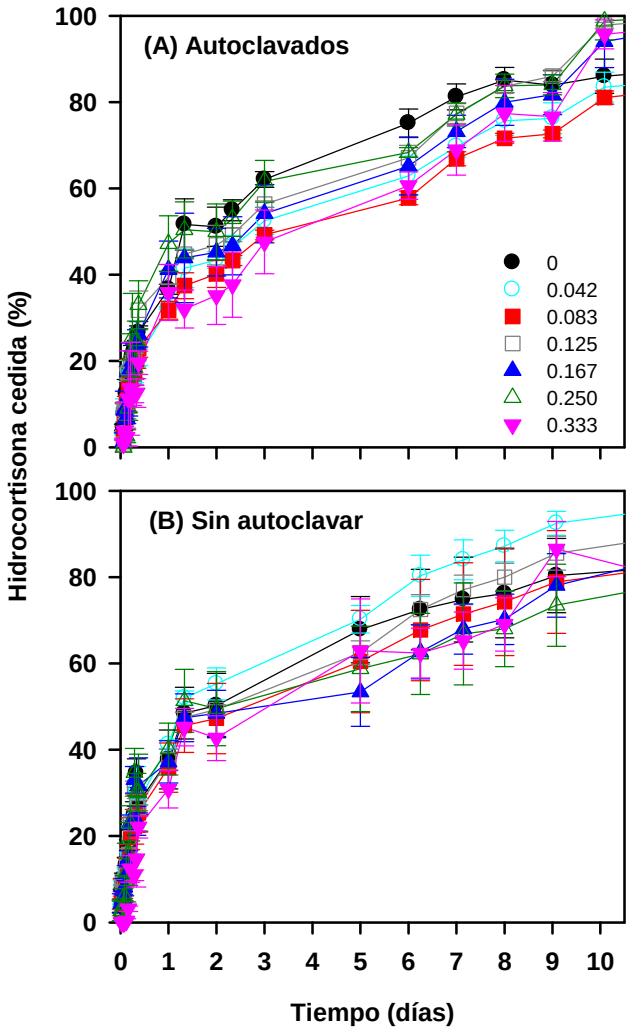

Fig. 4.14. Perfiles de cesión de hidrocortisona a partir de hidrogeles pHEMA-co-βCD que se autoclavaron (A) y los que no se sometieron a tratamiento térmico (B) durante el proceso de carga.

La acetazolamida se cedió más lentamente que la hidrocortisona. No obstante, en los tres primeros días se liberó una fracción importante de la dosis (Fig. 4.15), que corresponde al fármaco que se encuentra disuelto en la fase

acuosa del hidrogel o unido al entramado polimérico a través de interacciones débiles. A medida que aumenta la proporción de βCD, el tiempo que se requiere para la liberación se va incrementando hasta llegar a 24 días, como consecuencia de la formación de complejos de inclusión fármaco-βCD.

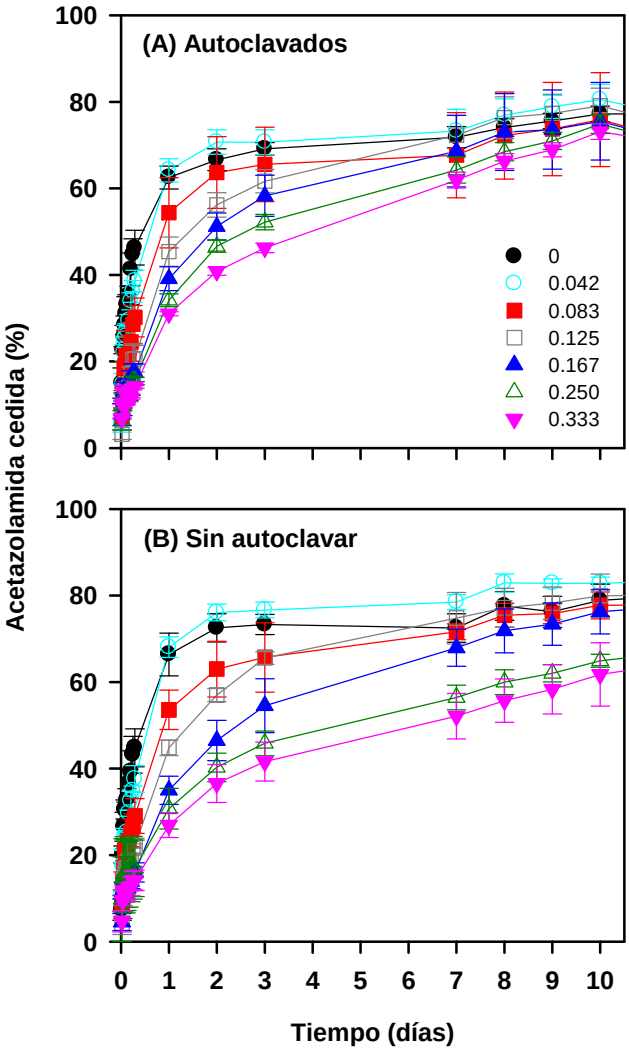

Fig. 4.15. Perfiles de cesión de acetazolamida a partir de hidrogeles pHEMA-co-βCD que se autoclavaron (A) y los que no se sometieron a tratamiento térmico (B) durante el proceso de carga.

En su conjunto, los resultados obtenidos en esta etapa del trabajo prueban que la copolimerización de βCD con HEMA da lugar a hidrogeles capaces de cargar hidrocortisona y acetazolamida y cederlos de manera sostenida. Estos sistemas proporcionan un control de la liberación más eficaz que el que se consigue con otras aproximaciones basadas en la incorporación de los fármacos al hidrogel formando parte de sistemas coloidales como nanopartículas o microemulsiones (*Gulsen, 2004; Gulsen y Chauhan, 2005; Gulsen y col., 2005*) y ofrece la posibilidad de modular los perfiles a necesidades específicas ajustando las proporciones de monómeros.

4.2. Hidrogeles sintetizados por reticulación de monómeros acrílicos y posterior anclaje de ciclodextrinas

La segunda etapa del trabajo se centró en el desarrollo de un nuevo procedimiento de anclaje de CDs en hidrogeles previamente formados. Con esta aproximación, se pretende obtener entramados con propiedades mecánicas independientes del contenido en CD, con el fin de ampliar el ámbito de aplicación de los hidrogeles como sistemas de liberación de medicamentos. En primer lugar, se diseñaron y sintetizaron hidrogeles acrílicos incorporando a su estructura un monómero con un grupo químico susceptible de unirse covalentemente a las CDs en una etapa posterior. Para ello se seleccionó el glicildilmetacrilato (GMA), que cuenta con un grupo epóxido capaz de formar enlaces éter con los grupos hidroxilo. Un procedimiento similar se ensayó previamente con éxito para anclar CDs en la superficie de fibras textiles (*Gawish y col. 2006*) y plásticos (*Hirotsu, 2006*).

4.2.1. Preparación de hidrogeles pHEMA-co-GMA con βCDs colgantes

Se partió de una disolución de EDGMA (8mM) en HEMA a la que se añadió AIBN (10mM). La proporción de agente reticulante se estableció de manera que los hidrogeles presenten propiedades mecánicas adecuadas para su utilización como componentes de lentes de contacto. Alícuotas de la mezcla de reacción se mezclaron con distintas cantidades de GMA para conseguir concentraciones finales de este monómero de 0, 50, 100, 150, 200, 300 y 400 mM. Las mezclas resultantes se transfirieron a moldes ortoédricos en los que se llevó a cabo la polimerización. Una vez que se completó el proceso, las láminas de hidrogel se cortaron en forma de discos. El anclaje de la βCD se llevó a cabo sumergiendo los discos previamente desecados, en medio agua/DMF con βCD, NaCl y NaOH durante 24 horas a 80ºC y sometiendo el conjunto a agitación suave. Finalmente, se aplicó a los discos un protocolo de lavado utilizando sucesivamente agua, etanol y tampón fosfato de pH 7.4 (Fig. 4.16).

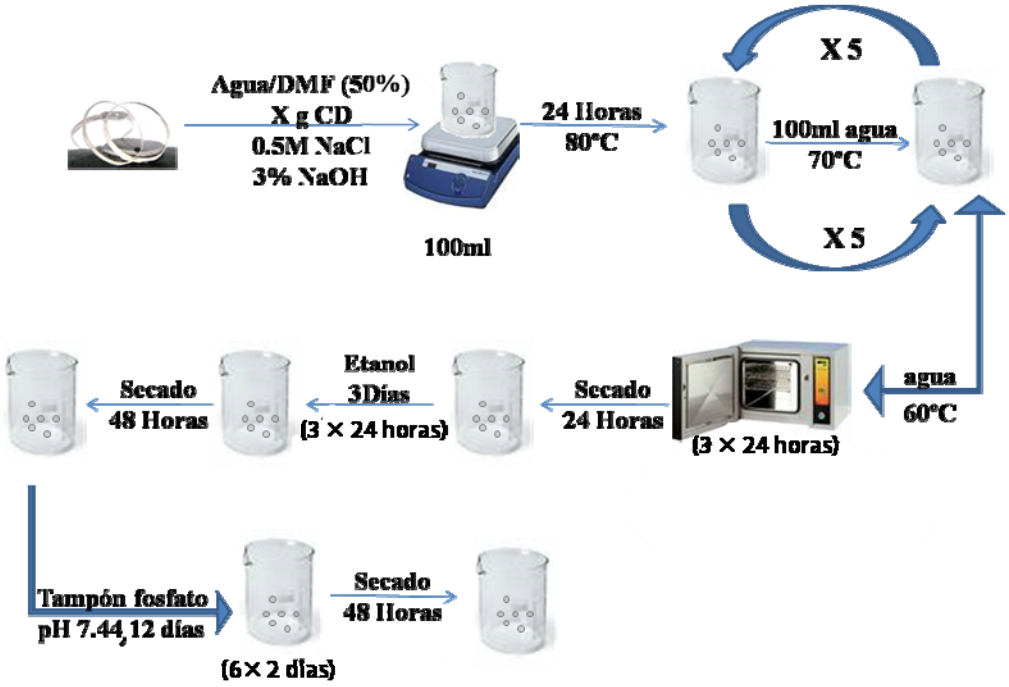

Fig. 4.16. Etapas del procedimiento de síntesis de los hidrogeles pHEMA-co-GMA con βCD colgante.

4.2.2. Caracterización de los hidrogeles

Los hidrogeles con las βCD ancladas mantuvieron el aspecto y la transparencia de los entramados de partida, con valores de transmitancia superiores al 90% a 600 nm. Los espectros de FT-IR (Fig. 4.17) no mostraron modificaciones relevantes tras el anclaje de la βCD, dado que las bandas características del grupo glicidilo, de la βCD y de los grupos éter del hidrogel de pHEMA se solapan a 1120cm^{-1}.

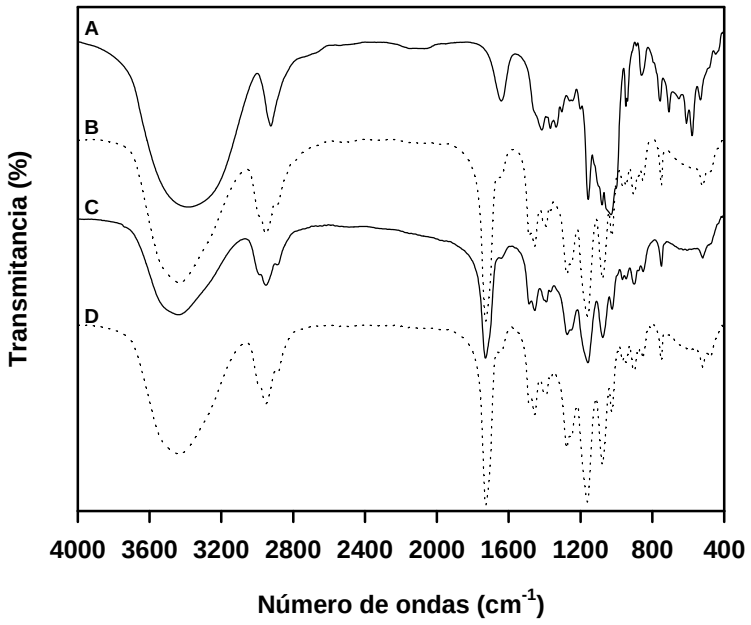

Fig. 4.17 Espectros de FT-IR de (A) βCD, (B) hidrogel control de pHEMA y (C) hidrogeles de pHEMA-co-GMA-βCD y (D) pHEMA-co-GMA sintetizados con la proporción más alta de GMA.

Para cuantificar el contenido en CDs ancladas en los hidrogeles se utilizó la técnica de captación de moléculas orgánicas de elevada afinidad, empleando como marcador 3-MBA. Los hidrogeles de pHEMA-co-GMA sin CDs colgantes sólo captaron pequeñas cantidades de 3-MBA y el contenido en GMA no afectó a la carga (Tabla. 4.6). A la vista de estos resultados se descartó el establecimiento de cualquier interacción entre los grupos glicidilo y el 3-MBA. La cantidad de 3-MBA cargada por los hidrogeles se incrementó progresivamente con el contenido en βCD colgante. Los resultados obtenidos indican también que los hidrogeles anclan una unidad de βCD por cada 2-3 grupos hidroxilo de GMA.

[GMA] (mM)	**GMA** (mmol/g)	**Carga 3-MBA antes de anclar βCD** (mg/g)	**Carga 3-MBA después de anclar βCD** (mg/g)	**βCD disponible** (mmol/g)	**GMA/βCD relación molar**
400	0.364	13.57 (0.93)	37.63 (1.70)	0.177	2.33
300	0.276	13.04 (1.18)	30.72 (0.86)	0.130	2.38
200	0.187	11.88 (0.51)	24.63 (3.14)	0.086	2.38
150	0.141	12.63 (0.18)	22.88 (0.59)	0.075	2.05
100	0.095	11.77 (0.68)	19.08 (1.19)	0.054	1.92
50	0.048	12.77 (0.34)	14.97 (0.34)	0.016	2.96
-	-	12.98 (0.38)	13.11 (0.15)	-	-

Tabla. 4.6. Cantidad de 3-MBA incorporada a los hidrogeles de pHEMA-co-GMA antes y después de anclar la βCD, cavidades de βCD disponibles para formar complejos de inclusión y proporción molar GMA/βCD en los hidrogeles con βCD anclada. Valores medios y, entre paréntesis, desviaciones estándar.

La distancia entre puntos de reticulación se estimó a partir del número de moléculas de agente reticulante EGDMA por unidad de volumen de hidrogel utilizando la expresión (*Alvarez-Lorenzo y col., 2000*):

$$R_X = \frac{10}{\sqrt[3]{[EGDMA]N_A}}$$

en la que N_A representa el número de Avogadro. Si se asume que el entramado está formado por celdas unidas en forma de pequeños cubos, la distancia entre los puntos de reticulación en el hidrogel seco será 5.92 nm. Cuando el hidrogel se hidrata, el volumen pasa a ser un 150% del inicial y el tamaño de malla se incrementa hasta 6.72 nm. Dado que una molécula de βCD tiene 1.53 nm de

ancho y 0.78 nm de profundidad (*Uekama y col., 1994*), no deben existir impedimentos estéricos que se opongan a la difusión de la βCD a través del entramado.

De manera análoga, la distancia entre grupos GMA estimada a partir del número de monómeros de GMA por unidad de volumen:

$$R_{GMA} = \frac{10}{\sqrt[3]{[GMA]N_A}}$$

resultó estar comprendida entre 3.21 nm, para el hidrogel preparado con GMA 50 mM y 1.6 nm para el hidrogel preparado con GMA 400mM (Tabla 4.7).

[GMA] mM	**R_{GMA}** (nm)
400	1.60
300	1.77
200	2.03
150	2.23
100	2.55
50	3.21

Tabla. 4.7. Distancia entre grupos GMA en el entramado.

Estos valores, que se incrementan en un 14% cuando el gel alcanza el equilibrio de hinchamiento, junto con las dimensiones de los grupos glicidilo del GMA y la flexibilidad que comunica a los hidrogeles la baja reticulación del

entramado, explican que una misma unidad de βCD pueda reaccionar simultáneamente con 2 ó incluso con 3 grupos glicidilo (Fig. 4.18).

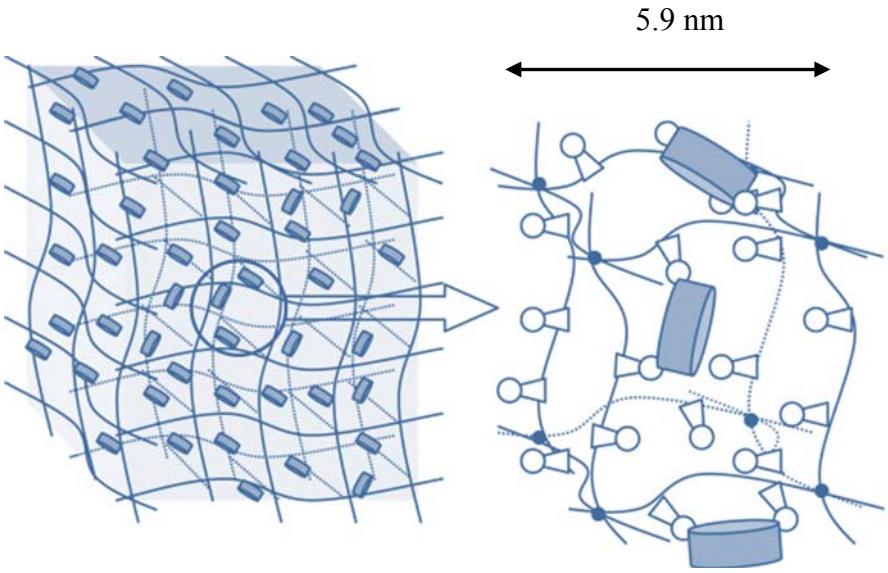

Fig. 4.18. Estructura de un hidrogel de pHEMA-co-GMA con βCD anclada.

El anclaje de la βCD no causó alteraciones en la temperatura de transición vítrea (T_g) de los hidrogeles (Tabla 4.8), lo que prueba que la βCD no modifica el grado de reticulación ni la rigidez del entramado. Los hidrogeles con un 2.5 mol% de βCD mantienen la Tg en 110ºC. Esta notable flexibilidad contrasta con la rigidez de los hidrogeles de pHEMA copolimerizados con 2,3-di-*O*-metacrilato-6-metacrilato-βCD, que con sólo 0.6 mol% de βCD presentan valores de T_g superiores a 150ºC. Desde un punto de vista práctico, este hecho tiene una gran trascendencia puesto que hace posible el incremento del número de cavidades de βCD disponibles para formar complejos de inclusión, sin alterar las propiedades mecánicas de los hidrogeles de partida. De esta manera la funcionalización con

CDs se puede compatibilizar con aplicaciones que, como la contactología, requieren materiales con elevada flexibilidad.

[GMA] (mM)	**GMA** (mmol/g)	**T_g** (ºC)
400	0.364	110
300	0.276	109
200	0.187	111
150	0.141	110
100	0.095	109
50	0.048	109
0	0	110

Tabla. 4.8. Temperatura de transición vítrea de los hidrogeles de pHEMA-co-GMA con βCD colgante.

Para caracterizar la hidrofilia superficial, se determinó el ángulo de contacto. La humectabilidad es otro factor crítico para que un hidrogel resulte útil como componente de lentes de contacto blandas, dado que ejerce una importante influencia sobre la biocompatibilidad y muy especialmente sobre la estabilidad del film lacrimal (*Tonge y col., 2004*). El ángulo de contacto aumentó ligeramente cuando HEMA se copolimerizó con proporciones elevadas de GMA, si bien su valor se redujo tras el anclaje de la βCD (Fig. 4.19) hasta situarse en valores similares a los del hidrogel control de pHEMA. A medida que transcurre el tiempo de ensayo, el ángulo de contacto se va reduciendo debido a los cambios conformacionales que se producen en la interfase pHEMA-agua; en el hidrogel seco los grupos hidrofóbicos se orientan hacia el aire pero, una vez que la

superficie se pone en contacto con el agua, se reordenan las cadenas exponiendo los grupos hidrofílicos hacia la interfase (*Senshu, 1995*).

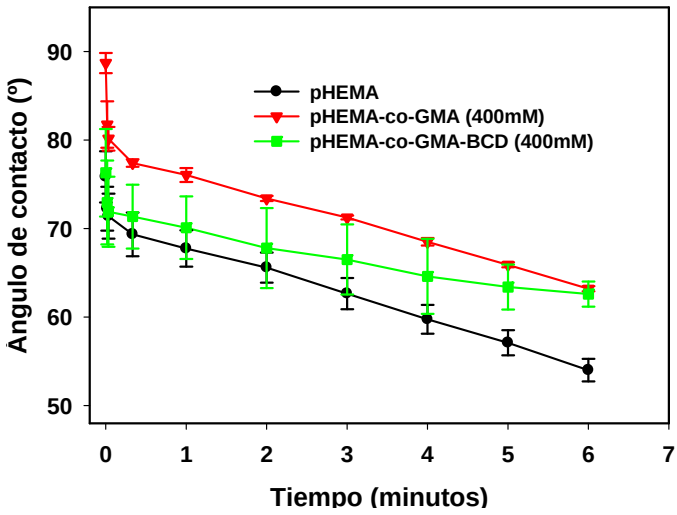

Fig. 4.19. Ángulo de contacto de los hidrogeles de pHEMA y de pHEMA-co-GMA antes y después de anclar βCD.

El anclaje de βCD no causó cambios relevantes ni en el grado ni en el tiempo necesario para alcanzar el equilibrio de hinchamiento (Fig. 4.20).

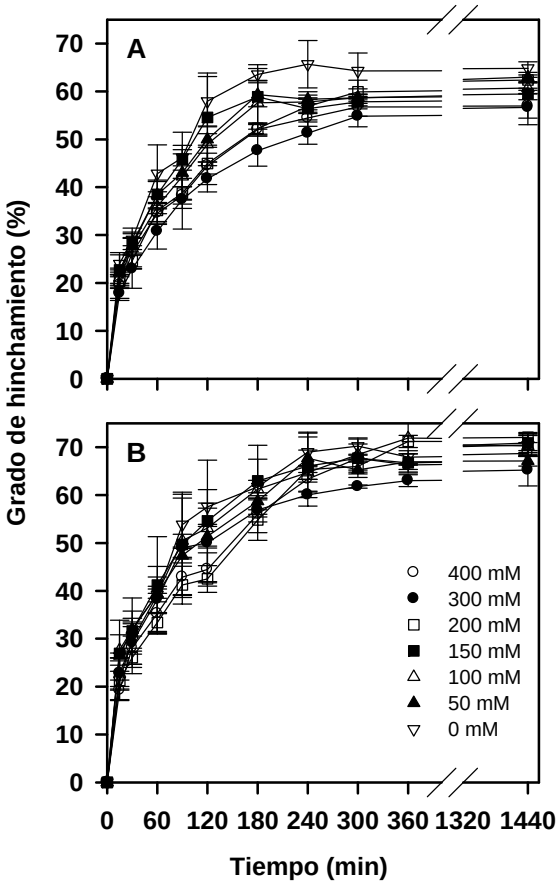

Fig. 4.20. Perfiles de hinchamiento de los hidrogeles de pHEMA-co-GMA preparados con distintos contenidos en GMA (valores en la leyenda) antes (A) y después (B) de anclar βCD.

Las propiedades viscoelásticas y de deslizamiento de las lentes de contacto hidratadas son determinantes para la sensación de confort una vez aplicadas en el ojo. Los módulos de almacenamiento (G') y de pérdida (G´´) de los hidrogeles con el contenido más alto en βCD colgante, determinados a 5 rad/s, fueron 80 KPa y 10 KPa, respectivamente. Estos valores se sitúan dentro del intervalo requerido

para que lentes de contacto blandas combinen una suficiente resistencia física con un grado de confort y una calidad visual adecuados (*Refojo y Leong, 1981*). Para cuantificar la facilidad de deslizamiento de los hidrogeles hidratados, se estimaron los coeficientes de fricción por un procedimiento reométrico, que se ha revelado más sensible y preciso que los procedimientos tribométricos (*Gong y col., 1999; Yañez y col., 2008*), dado que el ensayo se lleva a cabo con un control muy riguroso de la temperatura del material evaluado y la del líquido interpuesto entre su superficie y la del sólido de referencia (plato Peltier). Los ensayos se efectuaron a la temperatura de la superficie ocular (35ºC). El coeficiente de fricción (μ) de los hidrogeles sin βCD anclada fue de 0.35 ± 0.05, valor similar al encontrado previamente con hidrogeles de pHEMA para lentes de contacto blandas (*Yañez y col., 2008*) y con cartílagos sintéticos para articulaciones (*Freeman y col., 2000*). El anclaje de βCD redujo en un 50% el coeficiente de fricción (μ=0.15 ± 0.05) gracias al efecto deslizante de las ciclodextrinas colgantes y, en menor medida, al ligero incremento que producen en la hidrofilia superficial.

Para que el uso de los hidrogeles como lentes de contacto no cause edema ni hipoxia corneal, es necesario que presenten una suficiente permeabilidad al oxígeno. El anclaje de la βCD al entramado no alteró la permeabilidad de los hidrogeles de pHEMA, dando lugar a valores de 1.22×10^{-9} (cm^2/sec) (ml O_2/ml × mmHg) o 122 Barrer, que se sitúan dentro del intervalo que es habitual para lentes de contacto blandas de uso prolongado (*Gonzalez-Meijome y col., 2008*). Las diferencias encontradas entre hidrogeles con distintas proporciones de βCD anclada fueron inferiores al 5%.

4.2.3. Citocompatibilidad

La naturaleza de las estructuras que se combinan en los hidrogeles con βCD anclada hace previsible que estos materiales presenten una buena citocompatibilidad. Los monómeros que los constituyen mayoritariamente, HEMA y EGDMA, cuentan con la aprobación de las agencias reguladoras para ser utilizados en la síntesis de las lentes de contacto y otros productos sanitarios (*Lloyd y col., 2001; Alvarez-Lorenzo y Concheiro, 2006b*), y la βCD está considerada como un excipiente que no produce irritación a nivel ocular (*Cal y Centkowska, 2008*). No obstante, para descartar un hipotético efecto nocivo de monómeros residuales o dobles enlaces no reaccionantes, se evaluó la citocompatibilidad de los hidrogeles antes y después de anclar βCD utilizando macrófagos RAW 264.7. El cultivo se llevó a cabo en las condiciones descritas para los hidrogeles pHEMA-co-βCD. Al cabo de 5 días, se tiñó con calceina (células vivas) y con ioduro de propidio (células muertas) (Fig. 4.21). Con todos los hidrogeles se observó una excelente viabilidad celular. Los valores de IL-1α al cabo de 1, 5 y 10 días se situaron por debajo de los valores mínimos detectables (IL-1α 7.8pg/ml). Los niveles de TNF-α se detectaron a las 24 horas (1000 pg/ml) pero el marcador desapareció a partir de los 5 días. Por lo tanto, los hidrogeles son citocompatibles.

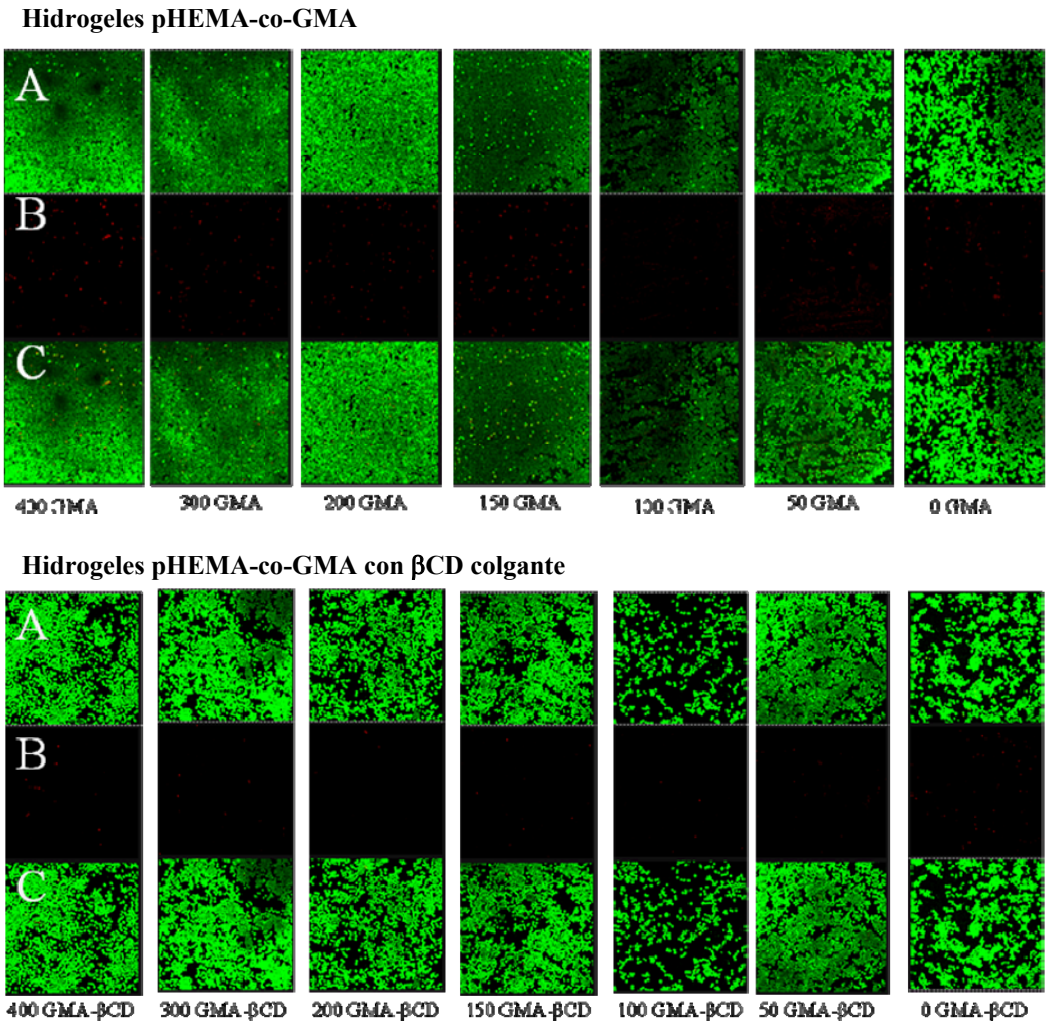

Fig. 4.21. Viabilidad de células RAW 264.7 al cabo de 5 días de contacto con hidrogeles pHEMA-co-GMA-βCD. (A) Tinción con calceína (células vivas); (B) tinción con ioduro de propidio (células muestras) y (C) superposición de las dos tinciones.

4.2.4. Carga y cesión de diclofenaco

El diclofenaco sódico (fig. 4.22) se utiliza frecuentemente en terapéutica ocular en el tratamiento sintomático de procesos inflamatorios y de conjuntivitis crónica no infecciosa (*Drug Facts and Comparisons, 2008*).

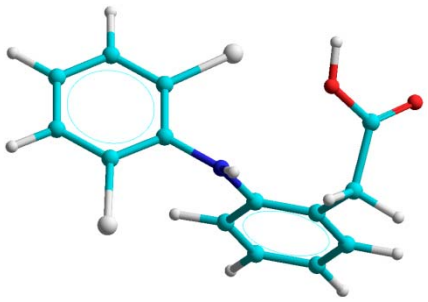

Fig. 4.22. Conformación espacial del diclofenaco.

El diclofenaco puede formar complejos de inclusión con αCD, βCD, HPβCD y γCD (*Fischer y Sendl-Lang, 1997; Arancibia y Escander, 1999; Pose-Vilarnovo y col., 1999; Arancibia y col. 2000; Pose-Vilarnovo y col., 2003; Abdoh y col., 2007*). La complejación tiene lugar por inclusión del anillo fenólico en la parte ancha de la cavidad de la βCD (Fig. 4.23).

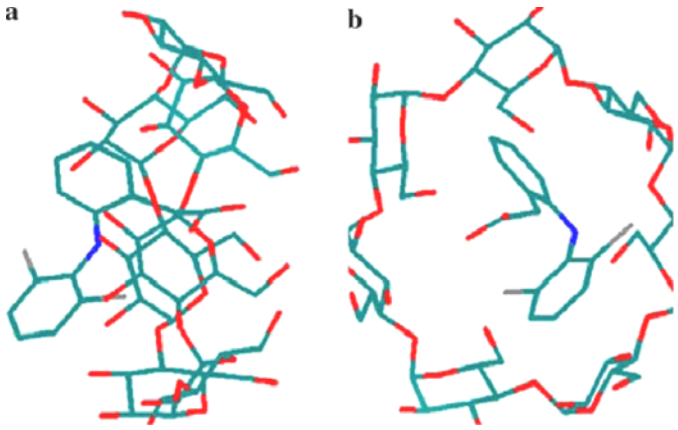

Fig. 4.23. Vista lateral (a) y frontal (b) de la estructura optimizada del complejo diclofenaco:βCD (Abdoh y col., 2007).

Los hidrogeles de pHEMA (sin GMA ni βCD) cargaron una cantidad pequeña de diclofenaco, disuelto en la fase acuosa del hidrogel y/o unido inespecíficamente al entramado polimérico (Tabla 4.9). Con la incorporación de βCD colgante, la capacidad de carga se multiplicó por 20, observándose un incremento progresivo al aumentar la proporción de βCD anclada (Tabla 4.9).

[GMA] (mM)	**Diclofenaco cargado (mg/g)**	
	Sin βCD	Con βCD
400	0.52 (0.06)	8.12 (0.58)
300	0.40 (0.06)	8.82 (0.54)
200	0.55 (0.11)	7.42 (0.75)
150	0.49 (0.09)	4.38 (0.21)
100	0.57 (0.05)	3.78 (0.65)
50	0.51 (0.11)	2.14 (0.15)
0	0.46 (0.09)	0.43 (0.14)

Tabla 4.9. Diclofenaco cargado por los hidrogeles de pHEMA-co-GMA con y sin βCD colgante a partir de una disolución de concentración 88.72 mg/l.

La cantidad de fármaco que se incorpora disuelto en la fase acuosa del hidrogel se estimó mediante la ecuación (*Kim y col., 1992*):

$$carga\ (fase\ acuosa) = \left(\frac{V_S}{W_p}\right) * C_0$$

en la que V_s representa el volumen de agua absorbida, W_p es el peso seco del hidrogel y C_0 la concentración inicial del fármaco en la disolución de carga. Con una absorción de agua de 0.6 ml/g (fig. 4.20), el fármaco incorporado en la fase acuosa (0.048 mg/g) es muy inferior a cualquiera de los valores que se observan en la tabla 4.10. Las diferencias entre la carga total y la debida al fármaco disuelto en la fase acuosa se explica por el establecimiento de interacciones hidrofóbicas con el entramado y a la formación de complejos de inclusión con las CDs colgantes a través de los anillos aromáticos. Para profundizar en el análisis de las interacciones diclofenaco/ciclodextrina, se calculó el coeficiente de reparto del fármaco entre el entramado polimérico y la disolución de carga (tabla 4.10) utilizando la siguiente ecuación (*Kim y col., 1992*):

$$carga\ (total) = [\frac{V_S + KV_P}{W_P} * C_0]$$

en la que V_p representa el volumen del hidrogel seco y los restantes símbolos mantienen el significado de la ecuación anterior.

[GMA] (mM)	**0**	**50**	**100**	**150**	**200**	**300**	**400**
Coeficiente de reparto	7	28	32	59	79	95	107

Tabla. 4.10. Coeficiente de reparto de diclofenaco entre el entramado polimérico y el agua en los hidrogeles de pHEMA-co-GMA con βCD anclada.

Los resultados obtenidos ponen de manifiesto la existencia de una correlación directa entre el valor de *K* y la proporción de GMA y, por lo tanto, la cantidad de βCD anclada en el hidrogel. La relación molar diclofenaco:βCD fue de 0.20-0.25, lo que indica que en los hidrogeles cargados permanecen libres entre el 70 y el 80% de las CDs. Las cantidades máximas que podrían llegar a incorporar los hidrogeles si se ocupase la totalidad de las cavidades de βCD se muestran en la tabla 4.11.

[GMA] (mM)	**0**	**50**	**100**	**150**	**200**	**300**	**400**
Contenido en βCD en el hidrogel (mmol/g)	0	0.016	0.054	0.075	0.086	0.130	0.177
Carga estequiométrica de diclofenaco (mg/g)	0	4.74	15.99	22.21	25.47	38.50	52.42

Tabla. 4.11. Contenido en βCD de los hidrogeles pHEMA-co-GMA con βCD colgante y cantidad máxima de diclofenaco que podría cargase si se ocupasen todas las cavidades de βCD presentes en el hidrogel.

Un aspecto muy relevante para la utilización práctica de las lentes de contacto medicadas es su aptitud para retener el fármaco cargado mientras se mantienen en los líquidos de conservación hasta el momento de su uso. Teniendo en cuenta este hecho, se introdujeron discos cargados de diclofenaco sódico en estuches individuales con 1 ml de líquido multifunción para lentillas (Multipurpose Solution Menicare Soft, Menicon Co. Ltd, Japón) y se conservaron herméticamente cerrados. Al cabo de un mes, se determinaron las cantidades de fármaco cedidas al líquido y remanentes en las lentillas. Los hidrogeles con βCD anclada mostraron una gran capacidad de retención de fármaco, siendo las

cantidades cedidas irrelevantes desde un punto de vista práctico (Fig. 4.24). Por lo tanto, estos materiales combinan una capacidad adecuada para cargar dosis terapéuticas de fármaco con una retención suficiente del fármaco cargado mientras las lentes se conservan en un líquido comercial de limpieza hasta ser usadas.

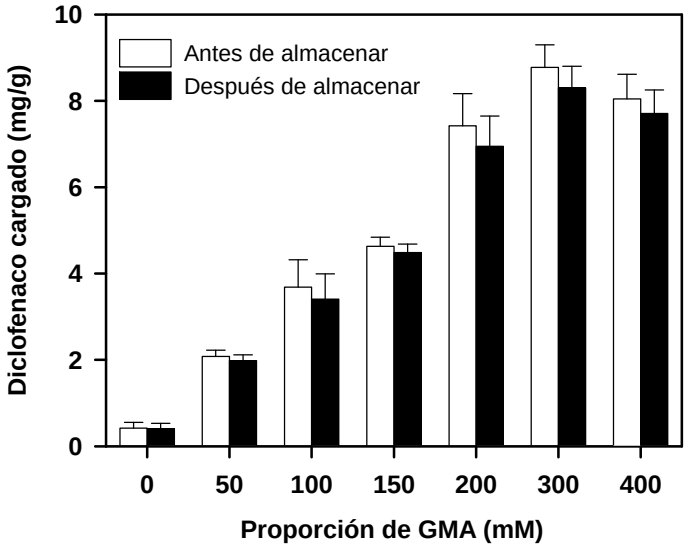

Fig. 4.24. Cantidad de fármaco en los hidrogeles de pHEMA-co-GMA con βCD colgante, antes y después de su almacenamiento durante un mes en un líquido comercial de conservación de lentillas (Menicare Soft Menicon Co. Ltd, Japón).

Los ensayos de cesión en fluido lacrimal mostraron una dependencia muy clara de la velocidad con que se libera el fármaco con respecto a la proporción de βCD anclada. Para un contenido determinado en βCD, los perfiles obtenidos con hidrogeles recién cargados y con hidrogeles almacenados durante un mes en líquido de conservación de lentillas fueron muy similares (Fig. 4.25). Los

hidrogeles de pHEMA (sin βCD) cedieron rápidamente toda la dosis por difusión completándose el proceso en un día, mientras que los hidrogeles con βCD anclada controlaron la cesión hasta dos semanas (Fig. 4.26). Esto confirma el importante papel de la decomplejación en el control de la liberación.

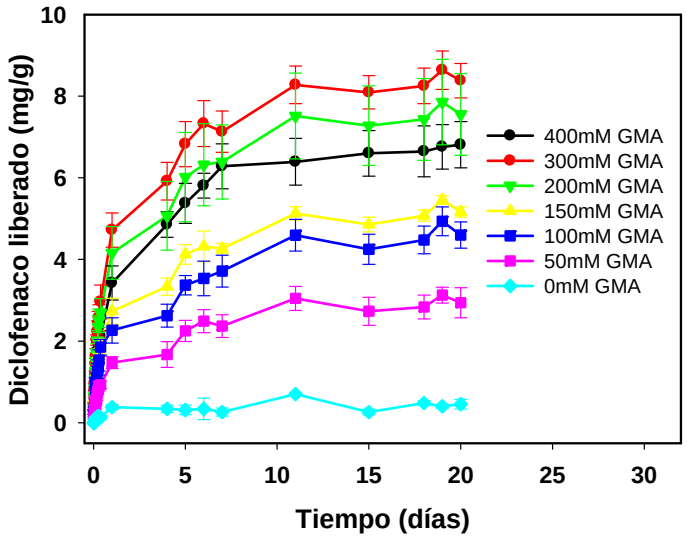

Fig. 4.25. Perfiles de cesión de diclofenaco a partir de hidrogeles de pHEMA-co-GMA con βCD colgantes recién cargados de fármaco.

La alta afinidad del fármaco por el hidrogel permite evitar que se produzca un efecto "burst", tal como se observa al ampliar la escala de tiempo correspondiente al primer día de ensayo de cesión (Fig. 4.26). Aunque los experimentos se llevaron a cabo en condiciones *sink*, la cantidad total cedida fue ligeramente inferior a la cargada, debido a que la afinidad del fármaco por el entramado hace que, en el equilibrio, una pequeña cantidad de éste permanezca unida al hidrogel. Sólo renovando el medio de cesión se pudo extraer el fármaco en su totalidad. Si estos hidrogeles se usasen para preparar lentes de contacto

medicadas, se podría conseguir una liberación muy sostenida teniendo en cuenta que el volumen de líquido en el área precorneal es muy reducido. La presencia de CD debe conducir a que se alcance un equilibrio entre el fármaco libre disponible para la absorción y el complejado con las CDs ancladas en el hidrogel. En el caso de que el fármaco no se absorbiese, hay que esperar que se interrumpa la cesión con lo que no se acumulará fármaco libre en la superficie de la cornea.

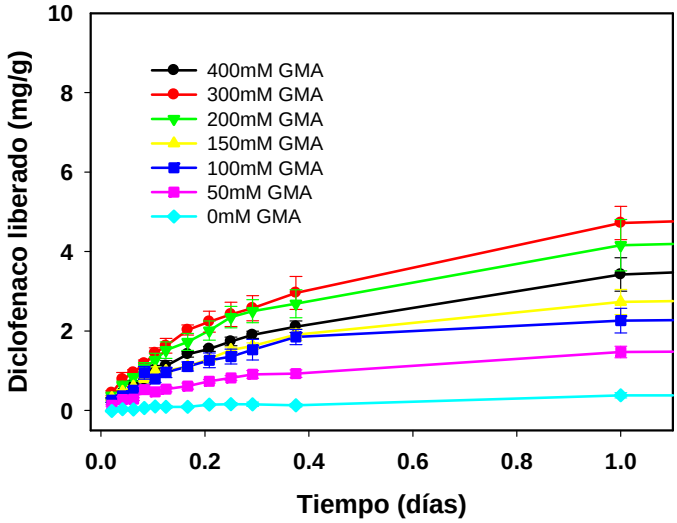

Fig. 4.26. Perfil de cesión de los hidrogeles de pHEMA-co-GMA con βCD colgante durante las primeras 24 horas.

4.3. Preparación de hidrogeles pHEMA-co-GMA con αCD y γCD colgantes

En la última etapa del trabajo se llevó a cabo un estudio dirigido a evaluar la influencia de la naturaleza de ciclodextrina colgante en las propiedades de los hidrogeles como sistemas de liberación de medicamentos. Para ello, se prepararon hidrogeles con αCD, βCD y γCD colgantes de manera similar a la descrita en el apartado 4.2.1. para los hidrogeles con βCD colgante. Para ello, se partió de una disolución de EDGMA (8mM) en HEMA a la que se añadió AIBN (10mM). Alícuotas de la mezcla de reacción se mezclaron con distintas cantidades de GMA para conseguir concentraciones finales de este monómero de 0, 100, 200, 300 y 400 mM. Las mezclas resultantes se transfirieron a moldes ortoédricos en los que se llevó a cabo la polimerización. Una vez que se completó el proceso, las láminas de hidrogel se cortaron en forma de discos. Los discos se dividieron en cuatro grupos: a) control, que no se sometieron a ningún tratamiento adicional; b) funcionalizados con αCD, que se sumergieron en medio agua/DMF con αCD, NaCl y NaOH durante 24 horas a 80ºC sometiendo el conjunto a agitación suave; c) funcionalizados con βCD, que se trataron como los discos (b) reemplazando αCD por βCD; y d) funcionalizados con γCD, que se trataron como los discos (b) reemplazando αCD por γCD. Finalmente, se aplicó a los discos un protocolo de lavado utilizando sucesivamente agua, etanol y tampón fosfato de pH 7.4. El anclaje de las CDs a los hidrogeles se confirmó mediante espectroscopia de resonancia magnética nuclear de ^{13}C.

Al igual que se había observado previamente para los hidrogeles con βCD colgante, la presencia de αCD o γCD incrementó muy ligeramente la hidrofília de

los hidrogeles, dando lugar a pequeños aumentos en el grado de hinchamiento y a una pequeña reducción del ángulo de contacto (Fig. 4.27).

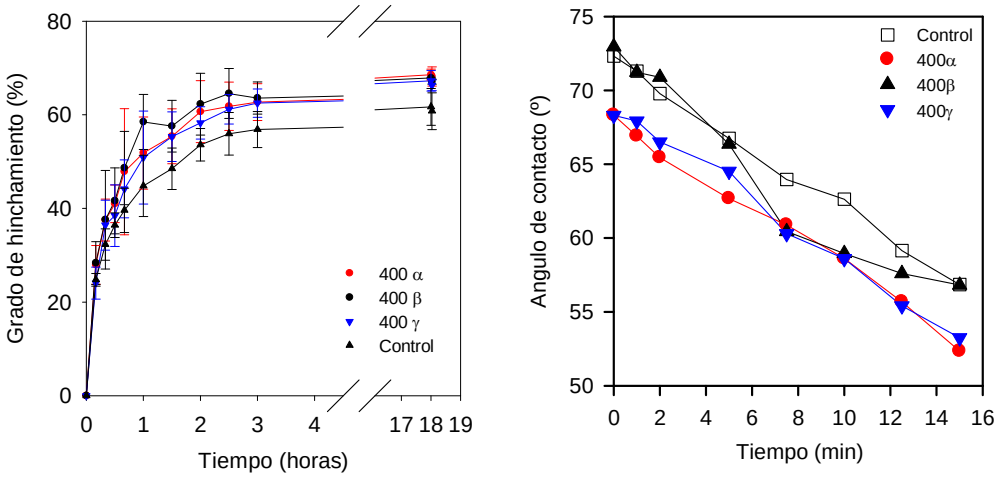

Fig. 4.27. Grado de hinchamiento y ángulo de contacto de los hidrogeles de pHEMA-co-GMA funcionalizados con la proporción más alta de αCD, βCD o γCD.

4.3.1. Adsorción de proteínas y citocompatibilidad

La tendencia de un biomaterial a adsorber proteínas determina en gran medida la naturaleza e intensidad de la reacción del organismo frente a lo que considera un cuerpo extraño (*Anderson y col., 2008*). Dado que los hidrogeles con CDs colgantes presentan unas propiedades mecánicas que los hacen aptos como implantes y como lentes de contacto, se llevó a cabo un estudio de adsorción con seroalbúmina, que es la proteína mayoritaria en sangre y en fluido lacrimal, y con lisozima, que actúa como antimicrobiano natural en el fluido lacrimal. Aunque las proteínas y otros componentes (por ej., mucina y lípidos) de las lágrimas pueden facilitar la humectabilidad de las lentes, se ha comprobado que, al adsorberse sobre las lentes de contacto, se desnaturalizan y se vuelven alergénicas

provocando ciertos síndromes oculares, como el "ojo rojo" y la conjuntivitis ocular (*Hume y col., 2004*). Estudios previos llevados a cabo con lentes de contacto basadas en pHEMA indican que la lisozima se puede adsorber superficialmente y también penetrar en la estructura de las lentillas, mientras que el mayor tamaño de la albúmina determina que sólo se adsorba sobre las lentillas (*Zainuddin y col., 2006*).

Los estudios de adsorción llevados a cabo en disoluciones monoproteínicas pusieron de manifiesto niveles más bajos de depósitos de proteínas en los hidrogeles con CDs colgantes que en los controles, excepto cuando se ancla αCD (Fig. 4.28). En todos los casos, el perfil de adsorción mostró una rápida deposición inicial seguida de un descenso más o menos marcado durante las 8 horas siguientes hasta que se estabiliza. Este tipo de perfiles son característicos de un gran número de biomateriales (*Zhang y col., 2005*). La funcionalización con γCD redujo marcadamente la tendencia de las proteínas a adsorberse sobre los hidrogeles. Los hidrogeles con βCD anclada ocuparon la segunda posición en cuanto a eficacia de prevención de la adsorción de proteínas, mientras que los funcionalizados con proporciones elevadas de αCD presentaron una elevada tendencia a adsorber albúmina y, en menor medida lisozima.

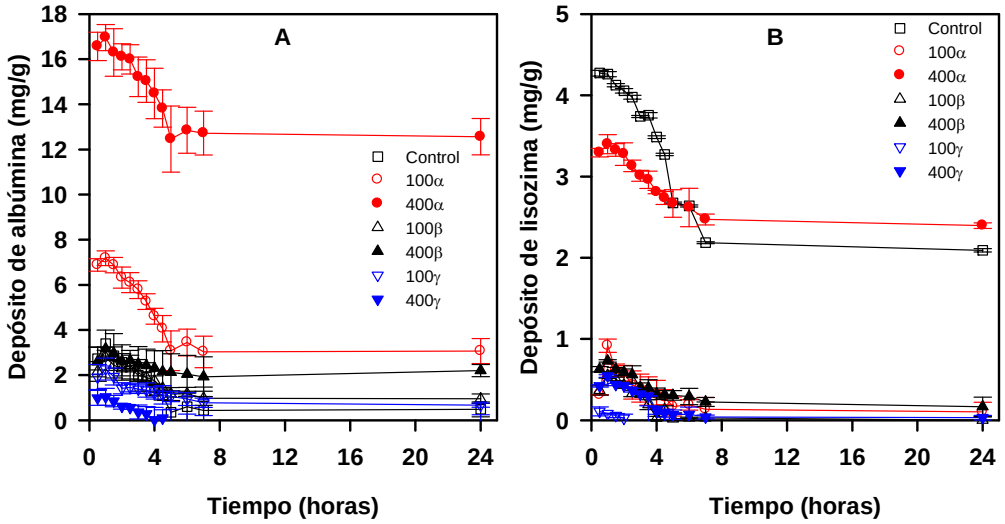

Fig. 4.28. Perfiles de adsorción de seroalbúmina (A) y de lisozima (B) de los hidrogeles de pHEMA-co-GMA funcionalizados con distintos niveles de αCD, βCD o γCD.

Estos resultados indican que, aunque las CDs incrementan ligeramente la hidrofilia superficial de los hidrogeles, la capacidad de la αCD y de la βCD para formar complejos de inclusión con algunos grupos hidrofóbicos de los aminoácidos (*Cooper y col., 1996; Yamamoto y col., 2006*) contribuye a la unión reversible de las proteínas sobre la superficie de los hidrogeles. Por su parte, las más bajas constantes de afinidad encontradas para la γCD explican que, a medida que se incrementa su presencia en los hidrogeles, la tendencia de las proteínas a depositarse en ellos se reduzca considerablemente.

Los estudios de citocompatibilidad llevados a cabo utilizando células Balb/3T3 clone A31, siguiendo las normas ISO10993-5:1999, pusieron de

manifiesto que todos los hidrogeles presentan una elevada citocompatibilidad *in vitro* (viabilidad celular > 95% después de 24 horas en contacto).

4.3.2. Carga y cesión de miconazol: inhibición de biofilms de *Candida albicans*

A continuación, se evaluó la capacidad de los hidrogeles para incorporar un fármaco tan hidrofóbico como el miconazol y proporcionar niveles locales eficaces en el tratamiento de infecciones por *Candida albicans*. Este microorganismo provoca procesos patológicos de elevada prevalencia en humanos y es responsable de la mayor parte de las colonizaciones por hongos de la superficie de los productos sanitarios (por ej., catéteres, prótesis, lentes de contacto,…) (*Danese, 2002; von Eiff y col., 2005*).

Una vez que se verificó que el miconazol es capaz de formar complejos de inclusión en medio acuoso con αCD, βCD o γCD con constantes de afinidad similares (436, 596 y 488 M^{-1}, respectivamente), los discos de hidrogel se sumergieron en suspensiones acuosas del fármaco y el conjunto se sometió a calefacción en autoclave para incrementar la solubilidad aparente del fármaco en agua y facilitar la penetración del fármaco en el entramado. Este tratamiento térmico, que no alteró las propiedades mecánicas de los hidrogeles, permitió obtener discos cargados de fármaco en menos de una hora. Inmediatamente después de autoclavar, la superficie de los hidrogeles se lavó con agua para arrastrar partículas de fármaco no incorporadas y los discos se desecaron.

Los perfiles de cesión de miconazol en agua se muestran en la figura 4.29. A pesar de que los hidrogeles hinchan rápidamente en agua, en el perfil de cesión se observó un tiempo de latencia de casi dos días, seguido de una liberación a velocidad prácticamente durante los seis días siguientes.

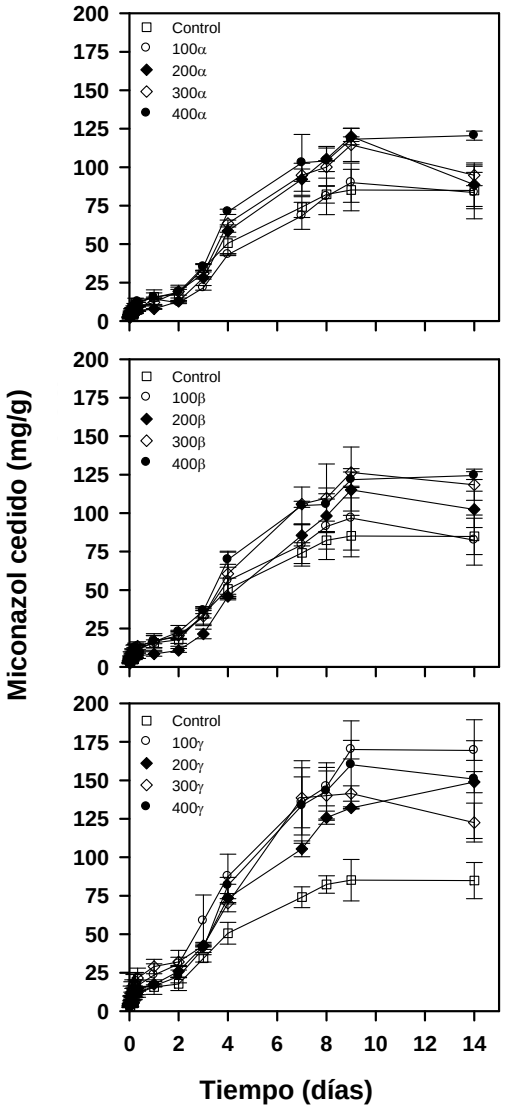

Fig. 4.29. Perfiles de cesión de miconazol a partir de hidrogeles de pHEMA-co-GMA funcionalizados con distintas proporciones de CDs colgantes.

Los perfiles de cesión no se asimilan a un proceso controlado por difusión, sino que parecen indicar una cinética de orden cero después de un tiempo de latencia. Aunque el miconazol es un fármaco muy hidrofóbico (solubilidad 0.17 mg/mL) (*Tenjarla y col., 1998*) y su disolución es lenta incluso en condiciones *sink*, no lo es tanto como para provocar el retraso observado en la cesión. Estos hechos sugieren que las interacciones fármaco-CD contribuyen al control del proceso de cesión. Por otra parte, en los perfiles se observa que los hidrogeles con γCD colgante proporcionan niveles de fármaco sensiblemente mayores a los de los demás hidrogeles.

En la Tabla 4.12 se recogen las cantidades totales de miconazol incorporadas en los hidrogeles, junto con las que se pueden alojar en la fase acuosa y las constantes de afinidad entramado-agua. Para todos los hidrogeles, la cantidad de fármaco incorporada en la fase acuosa del hidrogel fue muy baja dada su pobre solubilidad en agua. Los valores de carga total, prácticamente tres órdenes de magnitud mayores a los de la carga en la fase acuosa, indican que el fármaco presenta una elevada tendencia a establecer interacciones hidrofóbicas con el entramado de pHEMA. La afinidad de los entramados por el fármaco se incrementó al incorporar CDs colgantes. Los incrementos más relevantes se observaron para los hidrogeles con γCD anclada, que incluso duplicaron el coeficiente de reparto entramado-agua de los hidrogeles control.

Hidrogel	**Carga total de miconazol** (mg/g)	**Miconazol en la fase acuosa** (mg/g)	***K***	**Valores relativos de *K***
Control	84.78 (11.78)	0.103 (0.007)	498	1
100 α	83.49 (17.08)	0.120 (0.003)	490	0.98
200 α	88.56 (14.11)	0.120 (0.002)	520	1.04
300 α	94.75 (6.76)	0.117 (0.002)	557	1.12
400 α	120.50 (2.94)	0.116 (0.004)	708	1.42
100 β	82.40 (16.31)	0.119 (0.004)	484	0.97
200 β	102.40 (11.76)	0.122 (0.002)	602	1.21
300 β	118.35 (10.12)	0.119 (0.006)	696	1.40
400 β	124.32 (2.56)	0.114 (0.001)	731	1.47
100 γ	169.36 (6.41)	0.117 (0.004)	996	2.00
200 γ	148.78 (6.83)	0.119 (0.001)	874	1.75
300 γ	140.06 (12.64)	0.117 (0.005)	823	1.65
400 γ	150.76 (6.58)	0.113 (0.002)	886	1.78

Tabla 4.12. Cantidad de miconazol incorporada en los hidrogles (total y en la fase acuosa), coeficiente de reparto entramado-agua (K) y valores relativos de K referidos al hidrogel control de pHEMA.

Si se tiene en cuenta que la concentración mínima inhibitoria (MIC) de miconazol frente a *Candida spp.* es de 0.4-0.8 mg/L *(Piel y col., 1998)* y que, como promedio, un disco de 1 cm^2 incorpora 5-7 mg de fármaco, los hidrogeles son potencialmente capaces de evitar el crecimiento de *Candida spp.* en diez litros de agua. No obstante, dada la capacidad de *C. albicans* para dar lugar a biofilms de elevada resistencia antifúngica, tanto cuando colonizan mucosas como cuando

se establecen sobre la superficie de los biomateriales (*Chandra y col., 2001*), la siguiente etapa del estudio se centró en evaluar la capacidad para inhibir la formación de biofilms. Los ensayos microbiológicos consistieron en poner los discos en contacto, durante una hora, con un inoculo de *C. albicans* y, a continuación, cultivarlos durante 24 horas a 37ºC. Transcurrido este tiempo, se observó que los hidrogeles no cargados de fármaco estaban recubiertos por films constituidos por un elevado número de colonias de *C. albicans* (Fig. 4.30). Por el contrario, los hidrogeles capaces de incorporar las dosis más altas de miconazol presentaron un número de colonias muy bajo (4-5 reducciones décimas respecto al control). Incluso, algunos hidrogeles con γCD anclada y cargados con miconazol inhibieron por completo el crecimiento de *C. albicans*.

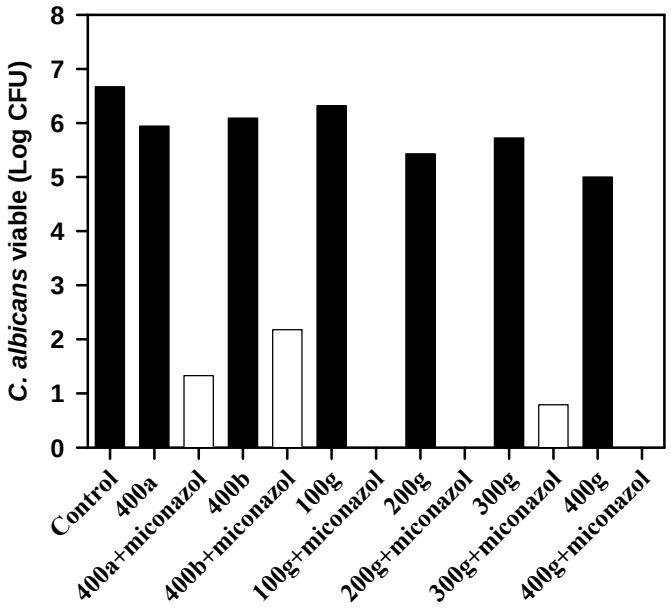

Fig. 4.30. Colonias de C. albicans integrantes de biofilms sobre la superficie de los hidrogeles de pHEMA-co-GMA funcionalizados con CDs colgantes, cargados o no con miconazol.

Los resultados de los ensayos microbiológicos sugieren que los hidrogeles funcionalizados con CDs y cargados de miconazol podrían ser útiles tanto para tratar infecciones fúngicas ya asentadas, como para prevenir el crecimiento de microorganismos sobre su superficie si se contaminan en el momento de su inserción. Por lo tanto, cuentan con potencial interés como componentes de sistemas de liberación útiles en terapéutica antifúngica y como componentes de productos sanitarios medicados con actividad profiláctica.

5. Conclusiones

5. CONCLUSIONES

Los resultados de los estudios dirigidos a desarrollar sistemas de liberación de medicamentos basados en hidrogeles acrílicos con ciclodextrinas incorporadas al entramado polimérico, obtenidos por aplicación de dos procedimientos diferentes, han permitido extraer las siguientes conclusiones.

1) En lo que se refiere a los hidrogeles de hidroxietil metacrilato (HEMA) copolimerizado con monómeros de ciclodextrina, se ha observado que:

a) El monómero 2,3-di-*O*-metacrilato-6-metacrilato-βCD presenta unas características adecuadas para la síntesis de este tipo de hidrogeles.
b) La copolimerización de HEMA con 2,3-di-*O*-metacrilato-6-metacrilato-βCD, en proporciones comprendidas entre 0.23 y 1.82 % mol, conduce a la obtención de hidrogeles transparentes, con un grado de conversión superior al 74% y una excelente citocompatibilidad, que no activan la respuesta de macrófagos.
c) El comportamiento del 2,3-di-*O*-metacrilato-6-metacrilato-βCD como agente reticulante conduce a que, a medida que se incrementa su proporción en los hidrogeles, los entramados adquieran mayor

rigidez, aumente su temperatura de transición vítrea y se reduzcan el grado de hinchamiento y el contenido en agua libre.

d) La selección de la proporción HEMA:2,3-di-*o*-metacrilato-6-metacrilato-βCD permite regular la cantidad de fármaco (hidrocortisona o acetazolamida) incorporada al hidrogel -tanto en forma libre como formando complejos de inclusión con la ciclodextrina- y controlar la velocidad de cesión, proporcionando una liberación sostenida en el tiempo.

2) En lo que se refiere a los de hidrogeles de HEMA a los que se incorpora, una vez sintetizados, ciclodextrinas colgantes se ha observado que:

a) El método desarrollado permite el anclaje, bajo condiciones suaves, de ciclodextrinas naturales sin tener que acudir a la modificación previa de su estructura. Cada unidad de ciclodextrina se une covalentemente al hidrogel formando enlaces éter con dos o tres unidades de glicidil metacrilato.

b) Los hidrogeles presentan una elevada citocompatibilidad, flexibilidad y transparencia.

c) El anclaje de αCD, βCD o γCD al entramado polimérico no provoca cambios significativos en la temperatura de transición vítrea, el grado de hinchamiento, el ángulo de contacto, la permeabilidad al oxigeno y el comportamiento viscoelástico de los hidrogeles.

d) La incorporación de βCD reduce el coeficiente de fricción de los hidrogeles en un 50%, permite incrementar la carga de diclofenaco hasta un 1300% y la afinidad fármaco-hidrogel unas 15 veces. Además, contribuye a evitar una cesión prematura de diclofenaco en los líquidos de conservación de las lentes de contacto y proporciona

perfiles de liberación sostenida, en fluido lacrimal artificial, al menos durante dos semanas.

e) El anclaje de βCD o γCD reduce la tendencia de la seroalbúmina y de la lisozima a adsorberse sobre los hidrogeles, lo que hace previsible que contribuya a incrementar la biocompatibilidad de los entramados en contacto con sangre o fluido lacrimal. Por el contrario, la αCD promueve la adsorción de las proteínas al formar complejos de inclusión reversibles con algunos de sus grupos hidrofóbicos.

f) La presencia de las ciclodextrinas colgantes incrementa la capacidad de los hidrogeles para incorporar un fármaco tan hidrofóbico como el miconazol. En concreto, la marcada tendencia del fármaco a la complejación con la γCD permitió duplicar la carga de los hidrogeles, dando lugar a sistemas capaces de inhibir completamente el desarrollo de biofilms de *Candida albicans*.

El análisis comparativo de las características de los dos tipos de hidrogeles desarrollados indica que, aunque las dos aproximaciones conducen a la obtención de sistemas citocompatibles capaces de incorporar fármacos y de cederlos de forma sostenida, el procedimiento basado en el anclaje de ciclodextrinas a hidrogeles preformados tiene la ventaja de que el entramado mantiene sus propiedades de partida cuando se incorporan las ciclodextrinas. Además, en este tipo de hidrogeles, al no formar parte las ciclodextrinas del entramado primario, no se producen impedimentos estéricos que puedan afectar a su capacidad de complejación con fármacos. En suma, los hidrogeles preparados aplicando cualquiera de las dos aproximaciones encierran un gran potencial como componentes de implantes o de lentes de contacto medicadas.

6. Bibliografía

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7. Otras publicaciones relacionadas con el trabajo de la Tesis

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Modulating drug release with cyclodextrins in hydroxypropyl methylcellulose gels and tablets

Beatriz Pose-Vilarnovo, Carmen Rodríguez-Tenreiro, José Fernando Rosa dos Santos, Juan Vázquez-Doval, Angel Concheiro, Carmen Alvarez-Lorenzo, Juan J. Torres-Labandeira*

Departamento de Farmacia e Tecnoloxía Farmacéutica, Facultade de Farmacia, Universidade de Santiago de Compostela, Campus Sur, E-15782 Santiago de Compostela, Spain

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Abstract

This paper reports on the effect of β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) on the diffusion and the release behavior of diclofenac sodium and sulphamethizole from HPMC K4M gels and matrix tablets. The gels were prepared with 0.5–2.0% polymer and different drug/CD mole ratios, and their viscosity, cloud point and drug diffusion coefficients were estimated. No differences in cloud point were observed. The viscosity of the gels strongly depended on HPMC proportions (from 0.7 to 100 mPa·s), which affected to a lesser extent the resistance to the diffusion of the drugs (D values from 60×10^{-6} to 5×10^{-6} cm^2/s). The influence of CD on diffusion was particularly evident in gels prepared with polymer proportions above its entanglement concentration, 2.0% HPMC K4M. In these systems, while high drug/CD proportions enhanced the diffusivity preventing polymer/drug hydrophobic interactions, low drug/CD ratios hindered it. An excess of free CD, especially the bulky HP-β-CD, made the diffusion of the complexes in the relatively low mesh size 2% polymer network more difficult. In the case of tablets, CD plays an additional role as dissolution rate promoter. To evaluate to what extent the balance between the increase in dissolution rate and the decrease in diffusion rate induced by CD determines drug release, matrix tablets were prepared by direct compression of 100 mg drug and 400 mg polymer/CD/lactose blends, whose composition was chosen following a simplex centroid design. A higher CD/lactose ratio significantly increased the release rate of hydrophobic drugs (sulphamethizole), but decreased the release rate of hydrophilic drugs (diclofenac sodium), indicating the predominance of a different contribution depending on the hydrophilicity of the drug. Therefore, the use of CD derivatives may be particularly useful to modulate drug release from HPMC gels and matrix tablets; the influence of these additives being dependent on the nature of the drug and on the molecular size and hydrophilic character of the CD used.

Keywords: Hydroxypropyl methylcellulose; Cyclodextrin; Diclofenac; Sulphamethizole; Hydrophilic matrices; Controlled release

1. Introduction

Preformed gels made of physically cross-linked polymers and matrix tablets, which form a gel layer when they come into contact with physiological

* Corresponding author. Tel.: +34-981-563-100; fax: +34-981-547-148.
E-mail address: ffjuant@usc.es (J.J. Torres-Labandeira).

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fluids, have been shown very useful as controlled release devices. Compared to other approaches, their relative easiness of manufacturing is complemented by the large variety of non-expensive polymers useful to provide viscose gels or to show high gelling capacities when enter in contact with aqueous medium [1].

Significant research has been done to try to establish the variables that determine the release behaviour from gels and matrix tablets in order to find new ways of achieving the required release pattern. In particular, the use of mixtures of polymers, especially cellulose ethers, has been shown particularly useful to regulate drug diffusion in gels [2]. In tablet matrices, excipient mixtures may modify not only the diffusion rate, giving gel barriers of varying consistency [3], but also the mechanism of drug release [4,5]. Most of these effects have their origin on the fact that the interactions between the excipients modify the microenvironment through which the drug has to diffuse out the matrix; in particular, its viscosity and polarity [2,6].

It is known that the ability of cyclodextrins to form inclusion complexes by taking up a whole drug molecule or rather some non polar part of it into the hydrophobic cavity may considerably alter drug diffusion properties in polymeric systems [7–9]. However, apparently, contradictory effects have been reported. For example, in cross-linked polyethylenglycol gels, nicardipine release rate was strongly decreased when cyclodextrin proportion increased [10]. On the other hand, Rao et al. [11] have observed that the presence of cyclodextrins considerably enhances the dissolution of prednisolone from 25% hydroxypropylmethylcellulose matrices; the higher the cyclodextrin/prednisolone ratio, the faster was the release. In general, if the drug is loaded in a concentration above saturation, cyclodextrins can accelerate the release by enhancing the proportion of diffusible species. When drug proportion is below the solubility limit, complexation reduces the concentration of free drug molecules. In this case, the overall effect on the diffusivity of the drug is not as foreseeable as before since it will depend on the diffusivity of the free species and of the complex, which is, in general, lower than that of the free drug. Aside from steric reasons, the diffusivity of cyclodextrin complexes may be limited by their solubility and also affected by the interactions with the polymeric components of the formulation [12]. Therefore, the use of CD derivatives of different solubility can provide different release rates [13]. If the complex cannot diffuse out of the matrix, a slow release of the drug would be achieved for complexes with high association constant.

To gain an insight into the factors that determine the role of cyclodextrins on drug diffusivity through preformed gels, and on the release rate from matrix tablets made of a commonly used hydrophilic polymer, HPMC K4M, we planned a study using two drugs with different water solubility and ionic charge, and two cyclodextrins differing in hydrophilicity and hydrodynamic size. Sulphamethizole (SUL) is a non-ionic drug practically insoluble (0.05 g/100 ml) [14] while diclofenac sodium (DIC) is an ionic drug ($pK_a = 4$) quite soluble (0.90 g/100 ml) in water at neutral pH [15]. The ability of cyclodextrins to form inclusion complexes with these drugs has been previously analysed by phase solubility techniques and ^{1}H-NMR spectroscopy. The halogenated aromatic ring of diclofenac and the benzene ring of sulphamethizol interact with the cavity of both β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD), preferentially forming 1/1 mol/mol complexes (stability constants: DIC-β-CD 100.6 M^{-1}; DIC-HP-β-CD 115.2 M^{-1}; SUL-β-CD 651.8 M^{-1}, SUL-HP-β-CD 563.9 M^{-1}) [16,17]. Cyclodextrin complexation considerably increases the solubility of these drugs in water.

The aim of this paper is to study how the characteristics of the drug and the cyclodextrin can condition the relative contribution of the different mechanisms involved in drug diffusion through the gels and the release from the matrix tablets, covering a wide range of HPMC proportions. The information obtained should also serve to establish criteria to adjust the composition of the formulations to the therapeutic requirements.

2. Materials and methods

2.1. Materials

Sulphamethizole (SUL) and diclofenac sodium (DIC) were obtained from Sigma (USA), hydroxypropyl methylcellulose (Methocel® HPMC K4M)

from Dow Chemical (USA), β-CD (molecular weight 1135, solubility in water 1.85 g/100 ml) and β-CD (4.6 d.s, solubility in water >60 g/100 ml) from Roquette-Laisa (Spain) and Janssen Pharmaceutical (Belgium), respectively, lactose from Merck (Spain) and magnesium stearate from J. Escuder (Spain).

2.1.1. Gels preparation and characterization

Gels were prepared by adding, under stirring, given amounts of HPMC K4M to diclofenac (50 mg/100 ml) or sulphamethizole (10 mg/100 ml) aqueous solution, containing or not cyclodextrin (1:0, 1:0.5, 1:1 and 1:3 drug/CD mole ratio). The dispersions were stored at 4 °C for 24 h for a complete swelling of the polymer and homogenisation of the systems before characterization.

2.1.2. Apparent viscosity

Cinematic viscosity measurements were carried out, by sextuplicate, in Cannon-Fenske capillary viscometers at 37 °C.

2.1.3. Cloud point

Cloud point (i.e., the temperature at which the transmittance is half that at room temperature) was determined in 1.0% HPMC dispersions, without and with 1.0% drug, containing 0.25%, 0.50% or 1.0% of each cyclodextrin, by measuring the transmittance at 800 nm in a Shimadzu UV-240 (Japan) spectrophotometer, at increasing temperatures (5 °C steps until close to cloud point, then 0.2 °C steps) [18].

2.1.4. Diffusion studies

Drug diffusion profiles from the different systems were obtained by triplicate in horizontal diffusion cells (Afora, España), thermostatted at 37 °C and fitted with cellulose acetate filters (0.45 μm pore size, Teknokroma, Spain) after storage at the same temperature for at least 1 h. The area available for diffusion was 0.7654 cm^2. The donor compartment contained 3 ml of gel, while the receptor compartment was filled with 3 ml deionized water. In both compartments, magnetic stirring (750 rpm) was applied. Samples of 0.5 ml were taken from the receptor medium at each predetermined sampling time, and immediately replaced with fresh medium. Diclofenac or sulphamethizole concentration in the receptor cell was determined by measurement of the absorbance at 276 nm ($E_{1\%,1\ cm}=283.85$) or 264 nm ($E_{1\%,1\ cm}=577.31$), respectively, in a Shimadzu UV-240 (Japan) spectrophotometer. Cyclodextrins did not interfere in the absorbance lecture.

Diffusion coefficients, D, were estimated using the equation [19]:

$$D = B^{*} l_m / C_1 \quad (1)$$

where B represents the slope of the release profile, l_m the thickness of the cellulose membrane and C_1 the initial concentration of drug in the gels.

2.2. Preparation and characterization of matrix tablets

Tablets containing 100 mg of diclofenac sodium or sulphamethizole were prepared, with 400 mg of polymer/cyclodextrin/lactose blends (Turbula T2C, Switzerland, 10 min) of different composition (Table 1), by direct compression in an instrumentalized Korsh Eko press (Germany) equipped with 12-mm flat punches, applying a compression force of 4.0–4.5 kN.

2.2.1. Dimensions

The dimensions of the tablets were measured by sextuplicate with a Mitutoyo (Japan) digital micrometer.

2.2.2. Friability

The friability of 10 tablets from each batch was determined using a Pharma Test (Germany) model PTF10E at 20 rpm for 15 min.

Table 1
Simplex centroid design used to prepare the tablet formulations

Formulation no.	x_1 (% HPMC)	x_2 (% CD)	x_3 (% Lac)
1	100	0	0
2	0	100	0
3	0	0	100
4	50	50	0
5	50	0	50
6	0	50	50
7	33.3	33.3	33.3
8	66.6	16.7	16.7
9	16.7	66.6	16.7
10	16.7	16.7	66.6

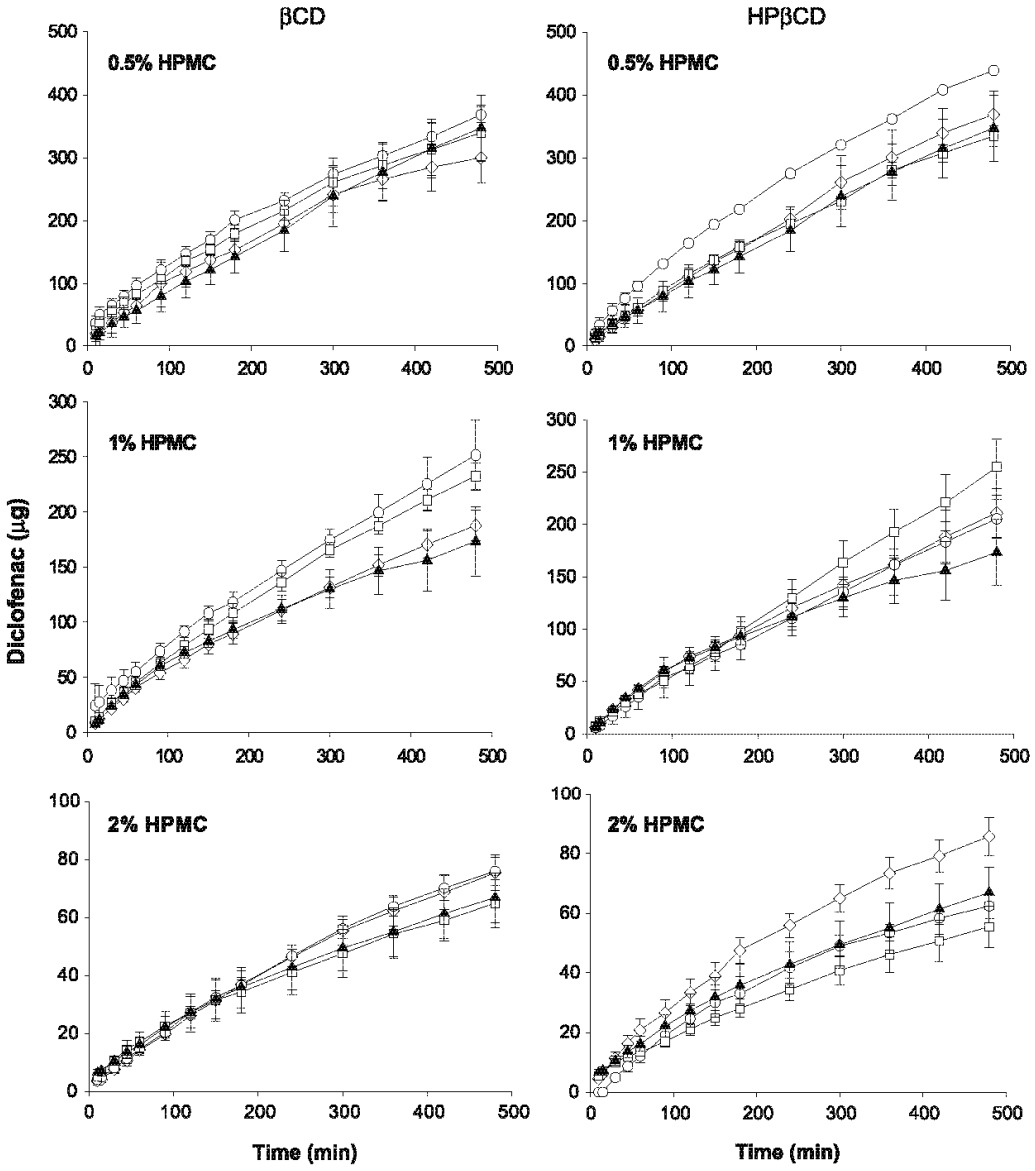

Fig 1. Diffusion profiles of diclofenac sodium from gels prepared with 0.5%, 1% and 2% of HPMC. Key: (▲) drug; (◇) drug–CD 1:0.5; (○) drug–CD 1:1; (□) drug–CD 1:3.

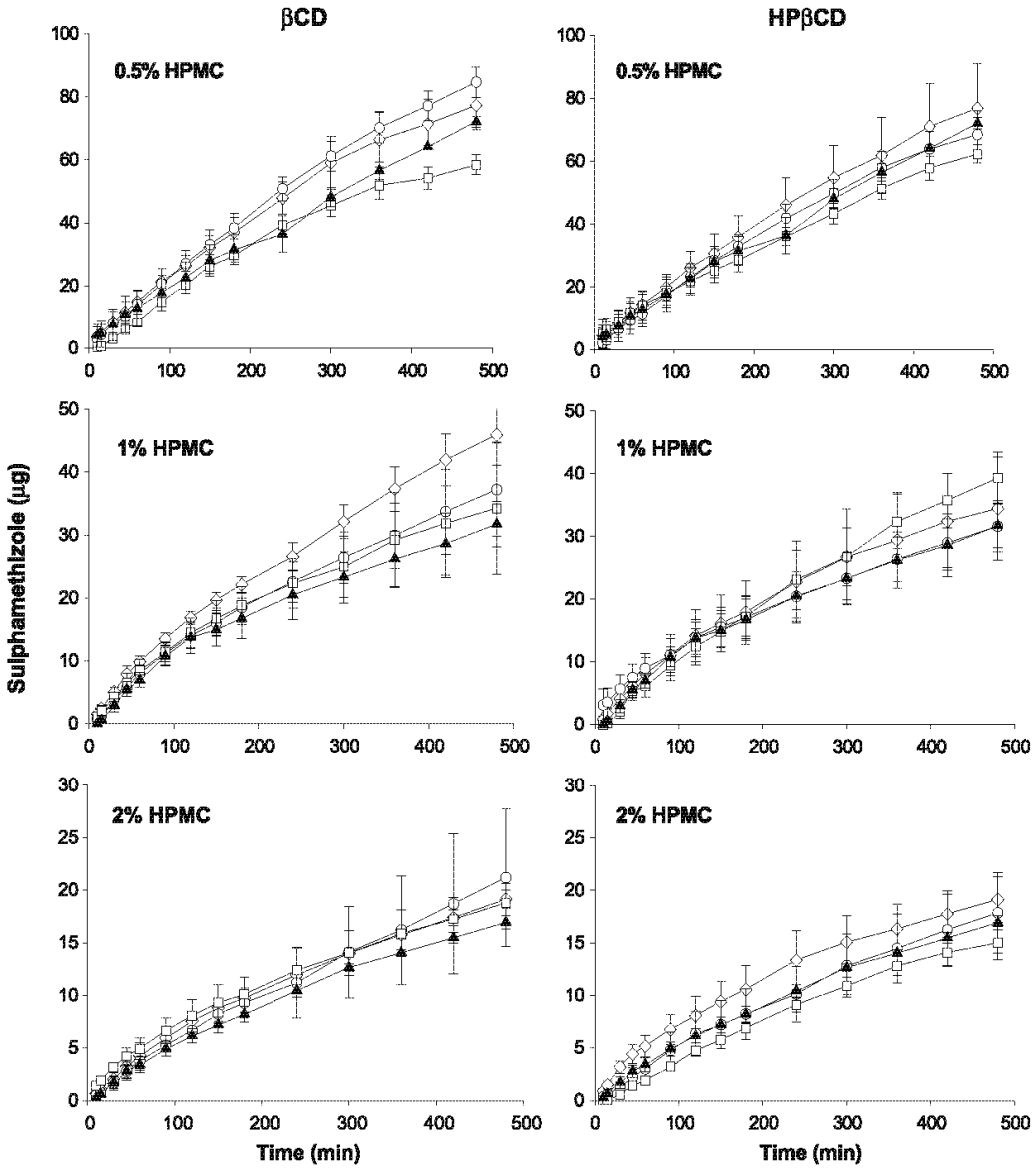

Fig 2. Diffusion profiles of sulphamethizole from gels prepared with 0.5%, 1% and 2% of HPMC. Key: (▲) drug; (◇) drug–CD 1:0.5; (○) drug–CD 1:1; (□) drug–CD 1:3.

2.2.3. Tensile strength

The tensile strength was calculated for each six tablets from the equation [20],

$$\text{Tensile strength} = 2\text{CS}/(\pi d \cdot e) \quad (2)$$

where CS is the crushing strength determined in an Erweka (Germany) TB-2A apparatus, d denotes the diameter of the tablet and e is its thickness (measured using a Mitutoyo digital micrometer).

2.2.4. Dissolution rate

Time-course of diclofenac and sulphamethizole release were determined at 37 °C in a Turu Grau (Spain) apparatus adapted to meet the specifications of the method II of USP 24 [21] . The tablets were introduced in small metallic baskets to avoid flotation and attachment to the vessel [22]. The dissolution medium was 900 ml of distilled water, stirred at 100 rpm. The concentration of the drug in 5 ml periodically taken samples was determined spectrophotometrically as described for the diffusion tests. The release profiles between 10% and 70% release were characterized by fitting the Higuchi equation [23]:

$$M_t/M_\infty = K_\text{H} \cdot t^{0.5} \quad (3)$$

where M_t corresponds to the amount of drug released in time t, M_∞ is the total amount of drug released after infinite time and K denotes a release rate constant.

2.3. Experimental design and statistical analysis

Gel composition followed a 4^2 factorial design in order to study the influence of HPMC proportion (0%, 0.5%, 1.0% and 2.0%) and the drug/CD mole ratio (1:0, 1:0.5, 1:1 and 1:3) on drug diffusion coefficient. The composition of the tablet formulations was adjusted to a simplex centroid design for blends of three variables at six levels each one (Table 1) [24]. Each formulation is designed by its number mixture, followed by B (when X_2 was β-CD) or H (when X_2 was HP-β-CD), and the letters DIC or SUL depending if the formulation contains diclofenac or sulphamethizole.

To quantify the effect of the variables analysed on the K_H, a step-wise linear multiple regression ($\alpha < 0.05$) was used (Statgraphics® v.7.0). Applying this procedure, in the case of simplex centroid design, the parameters of the second-order canonical equations:

$$y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (4)$$

and the reduced cubic-order canonical equations were obtained:

$$y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \quad (5)$$

where the variables x_1, x_2 and x_3 represent the percentage of HPMC, cyclodextrin and lactose in the mixture, respectively, and the coefficients β_1, β_2 and β_3 equal to the values of the response property in question for the pure HPMC, cyclodextrin or lactose tablet [24]. To decide whether to use the second-order canonical model or the cubic model, we calculated the Akaike Information Criterion (AIC) [25]:

$$\text{AIC} = N\ln\text{RSS} + 2p \quad (6)$$

where N is the total number of data points, RSS the residual sum of squares and p the number of parameters

Table 2
Diffusion coefficient ($\times 10^6$) and viscosity of diclofenac in HPMC gels; mean value (standard deviation), $n = 3$

	0% HPMC		0.5% HPMC		1% HPMC		2% HPMC	
	D ($\text{cm}^2\ \text{s}^{-1}$)	η (mPa s)	D ($\text{cm}^2\ \text{s}^{-1}$)	η (mPa s)	D ($\text{cm}^2\ \text{s}^{-1}$)	η (mPa s)	D ($\text{cm}^2\ \text{s}^{-1}$)	η (mPa s)
DIC	64.8 (6.9)	0.70 (0.00)	31.6 (4.3)	12.8 (0.4)	14.8 (1.0)	78.3 (1.2)	6.1 (1.0)	973 (24)
DIC/β-CD 1/0.5	63.4 (0.4)	0.74 (0.00)	30.8 (6.4)	9.9 (0.0)	17.4 (1.4)	70.9 (0.9)	7.4 (0.8)	881 (16)
DIC/β-CD 1/1	63.4 (1.3)	0.71 (0.00)	32.8 (2.0)	10.2 (0.2)	21.5 (1.0)	71.2 (2.2)	7.5 (0.2)	1020 (3)
DIC/β-CD 1/3	67.4 (8.1)	0.71 (0.00)	31.8 (4.7)	11.8 (0.2)	21.8 (0.9)	77.4 (1.8)	5.9 (1.0)	1010 (21)
DIC/HP-β-CD 1/0.5	61.1 (6.2)	0.71 (0.00)	35.5 (4.8)	9.8 (0.0)	19.0 (3.6)	70.9 (1.7)	8.6 (0.5)	895 (58)
DIC/HP-β-CD 1/1	61.6 (4.8)	0.71 (0.00)	43.3 (1.4)	10.8 (0.1)	18.7 (1.4)	81.9 (0.6)	6.9 (0.4)	1080 (6)
DIC/HP-β-CD 1/3	65.8 (4.4)	0.71 (0.00)	31.7 (0.4)	12.7 (0.1)	22.3 (2.9)	72.1 (2.0)	5.0 (0.8)	1160 (7)

fitted. In each case, the model selected was that which gave the lowest AIC value. The data for the contour plots were generated using MATLAB 6.5 (The MathWorks, Natick, MA).

3. Results and discussion

3.1. Gels

The presence of drug and cyclodextrin did not significantly modify the cloud point of 1% HPMC K4M dispersions, which was around 72.1 ± 0.3 °C in all gels. Changes in this parameter are related to a competition of additives with the polymer for water and with changes in conformation of its chains, which may affect the diffusion processes [26]. The data obtained indicate that the hydrophilicity and water "structure" around the cellulose ether did not experiment significant changes in the systems analysed. Additionally, since the concentrations of diclofenac sodium and sulphamethizole in the gels were lower than their solubility coefficient in water, the effect of drug complex formation with cyclodextrin should be mainly on the diffusivity through the HPMC network.

The amount of diclofenac (Fig. 1) or sulphamethizole (Fig. 2) diffused on a given time strongly decreased as the proportion of HPMC increased. This effect becomes especially evident when calculating the diffusion coefficients corresponding to each gel (Tables 2 and 3); an exponential decay as a function of HPMC concentration is observed [27]. It is also interesting to note that in the absence of HPMC, all solutions had similar diffusion coefficients irrespectively of the nature of the drug and the cyclodextrin and also of the proportion of cyclodextrin incorporated; significantly lower diffusion coefficients were only observed for sulphamethizole in the presence of the greatest HP-β-CD proportion. Increasing HPMC concentration, high drug/CD proportions tend to promote drug diffusion, while the opposite effect is observed for low drug/CD ratios. This may be explained in terms of the mesh size of the polymer network and the possible interactions between the components of the gel. HPMC concentrations selected cover a wide range of intermolecular association states. HPMC K4M intrinsic viscosity in water at 20 °C was previously estimated to be around 7.3 dl/g [2]. This means that its overlapping concentration is approximately 0.14 % and its entanglement concentration is slightly below 1% [28]. Therefore, it can be expected that the effect of the hydrodynamic size of the diffusant will be particularly evident in the lowest mesh size and the greatest tortuosity 2% HPMC systems. On the other hand, diclofenac sodium is an amphiphilic molecule that, although completely ionised under the experimental conditions in which the studies were carried out, can establish hydrophobic interactions with the less polar regions of cellulose chains [29]. The high hydrophobicity of sulphamethizole suggests a similar behavior. Therefore, their complexation with hydrophilic CD should prevent such interaction making the diffusion easier. This explains the fact that, in general, drug/CD 1:1 formulations in 0.5% and 1% HPMC gels, and drug/CD 1:0.5 in 2% HPMC gels, show the greatest diffusion coefficients. In contrast, an excess of CD may contribute to increase the tortuosity of the diffusional path of the drug/CD complexes, especially the bulky HP-β-CD in the dense 2% HPMC systems (Tables 2 and 3). The one-way ANOVA test indicated the existence

Table 3
Diffusion coefficient ($\times 10^6$) and viscosity of sulphamethizole in HPMC gels; mean value (standard deviation), $n=3$

	0% HPMC		0.5% HPMC		1% HPMC		2% HPMC	
	D ($cm^2\ s^{-1}$)	η (mPa s)	D ($cm^2\ s^{-1}$)	η (mPa s)	D ($cm^2\ s^{-1}$)	η (mPa s)	D ($cm^2\ s^{-1}$)	η (mPa s)
SUL	66.4 (5.2)	0.71 (0.00)	31.6 (1.1)	9.2 (0.0)	15.9 (2.9)	69.8 (1.9)	8.5 (0.1)	878 (13)
SUL/β-CD 1/0.5	60.9 (5.2)	0.71 (0.00)	38.9 (4.6)	9.7 (0.3)	21.6 (2.5)	67.3 (1.3)	9.3 (0.3)	940 (11)
SUL/β-CD 1/1	64.9 (6.4)	0.74 (0.00)	41.8 (3.9)	10.1 (0.1)	17.9 (3.5)	72.9 (0.4)	9.6 (3.5)	930 (19)
SUL/β-CD 1/3	60.4 (7.2)	0.71 (0.00)	32.8 (2.4)	9.6 (0.0)	17.1 (3.5)	73.5 (2.2)	8.9 (1.4)	722 (4)
SUL/HP-β-CD 1/0.5	66.4 (1.6)	0.73 (0.00)	36.3 (6.4)	9.9 (0.1)	17.7 (4.7)	77.7 (0.9)	9.6 (1.6)	796 (15)
SUL/HP-β-CD 1/1	55.6 (4.4)	0.71 (0.00)	34.6 (3.7)	10.1 (0.1)	14.3 (1.8)	70.6 (0.2)	8.7 (1.9)	766 (23)
SUL/HP-β-CD 1/3	49.4 (5.2)	0.70 (0.00)	27.5 (1.2)	9.5 (0.0)	19.6 (2.9)	65.8 (0.6)	7.9 (0.6)	825 (10)

of statistically significant effect ($\alpha < 0.05$) of the proportion of CD on the drug diffusion coefficient, except to the SUL in 2% HPMC gels.

The presence of 2% HPMC K4M in the drug dispersions increased the viscosity 1400 times. On the other hand, the microviscosity which provides information about the resistance to the drug diffusion and estimated as [2]:

$$\eta = \eta_0 \frac{D_0}{D} \quad (7)$$

where η_0 is the viscosity of water, and D and D_0 represent the drug diffusion coefficients in the gel and in water, respectively, increased less than 10 times (Tables 2 and 3). Additionally, while the differences in apparent viscosity, or macroviscosity, for a given percentage of HPMC are almost null, the changes that occur in the microenvironment through which the drug diffuses are, depending on the cyclodextrin proportion, very remarkable as shown by the different diffusion coefficients obtained. This highlights the limitations of using just macroviscosity to predict drug diffusion behavior through polymer dispersions [6].

3.2. Tablets

To prepare the matrix tablets a simplex centroid experimental design was followed (Table 1). Despite having a similar weight (0.500 ± 0.020 g), tablets differed in thickness (between 3.6 and 4.5 mm), friability and tensile strength. The formulations with greatest proportions of lactose (mixtures no. 3 (100%)

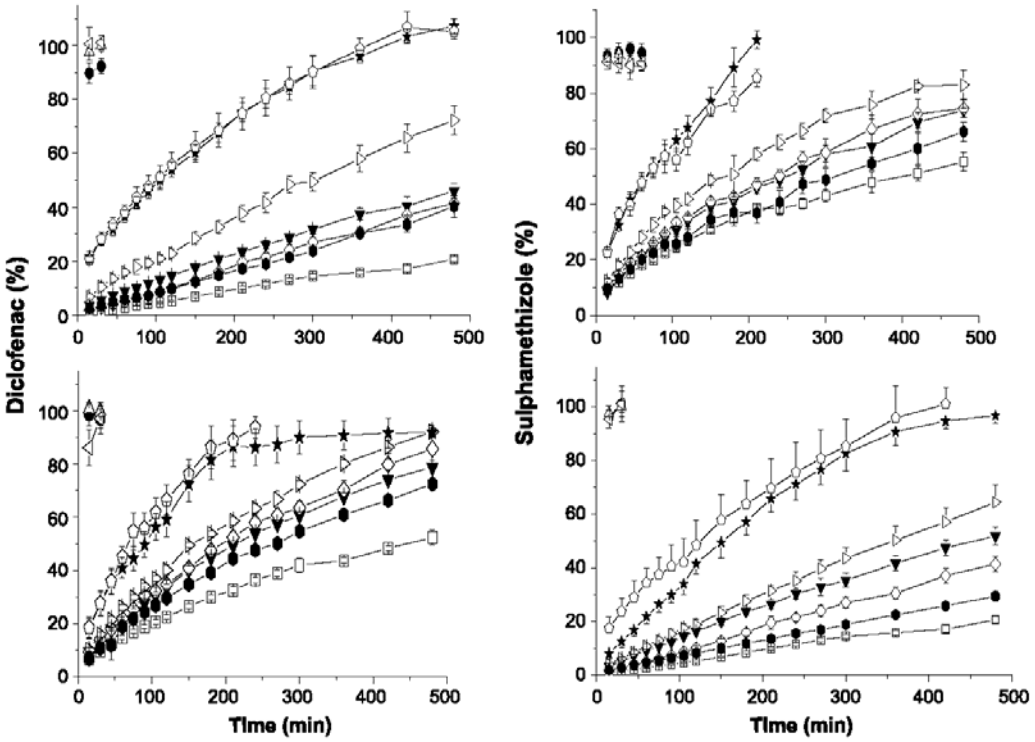

Fig 3. Dissolution profiles of the formulations prepared with diclofenac sodium or sulphamethizole and β-CD or HP-β-CD according to the experimental design (Table 1). Key: (□) no. 1; (●) no. 2; (△) no. 3; (▼) no. 4; (◇) no. 5; (◁) no. 6; (▷) no. 7; (⬟) no. 8; (★) no. 9; (○) no. 10.

Table 4
Drug release rate constant of the tablets elaborated with diclofenac sodium and HPMC/CD/lactose in the ratios shown in Table 1

Formulation	Experimental K_H (%·min$^{-0.5}$)	Predicted K_H (%·min$^{-0.5}$)	Formulation	Experimental K_H (%·min$^{-0.5}$)	Predicted K_H (%·min$^{-0.5}$)
1B-DIC	2.62 (0.18)	2.62	1H-DIC	2.63 (0.09)	2.75
2B-DIC	–	–	2H-DIC	–	–
3B-DIC	–	–	3H-DIC	–	–
4B-DIC	3.65 (0.15)	3.65	4H-DIC	4.04 (0.08)	4.15
5B-DIC	3.74 (0.23)	3.75	5H-DIC	4.36 (0.18)	4.48
6B-DIC	–	–	6H-DIC	–	–
7B-DIC	4.18 (0.14)	4.20	7H-DIC	4.71 (0.07)	5.06
8B-DIC	3.09 (0.11)	3.07	8H-DIC	3.76 (0.06)	3.29
9B-DIC	4.96 (0.23)	4.95	9H-DIC	6.58 (0.46)	6.46
10B-DIC	5.40 (0.15)	5.40	10H-DIC	7.09 (0.49)	6.97

The results of the statistical analysis are shown in Fig. 4.

and no. 10 (66.66%)) showed the greatest friability and lowest tensile strength; this effect was particularly evident in the case of sulphamethizole tablets, which friability (up to 50%) was the only above the Pharmacopoeia limit [21]. Tablets prepared with no HPMC (nos. 2, 3 and 6) did not behave as matrix systems, rapidly disintegrating and releasing in the first 15 min all drug loaded (Fig. 3). In contrast, the tablets containing just HPMC as excipient showed the most sustained profile. Similar results were observed for the release of adinazolam mesylate from HPMC K4M/lactose tablets [5]. Lactose is a soluble excipient that facilitates the penetration of water inside the tablet and, therefore, widely used to enhance the release rate of sparingly soluble drugs. When the proportion of lactose or other hydrosoluble excipients is high, tablets disintegrate quickly. This effect has been reported for carbamazepine/β-CD in HPMC K100LV matrices containing less than 15% of polymer [30].To the best of our knowledge, no study with ternary mixtures polymer/CD/lactose has been carried out yet.

Incorporation of cyclodextrins and lactose in different proportions provided a way of modulating drug release profiles, which were well fitted by the Higuchi equation [23] (correlation coefficients, r^2>0.97). The values of the rate constant obtained, summarized in Tables 4 and 5, were statistically different (one-way ANOVA test, $\alpha < 0.01$). The application of a multiple range test (Statgraphics® v.7.0) indicated that the formulations 4B-DIC and 5B-DIC have not statistically different release rates. In the case of sulphamethizole, release rates from formulations 9B-SUL and 10B-SUL, and from 5B-SUL and 8B-SUL, were not significantly different ($\alpha < 0.01$).

The plots of K_H versus the percentage of HPMC (Fig. 4) evidence the strong influence of this variable on drug release rate; the decrease in K_H being expo-

Table 5
Drug release rate constant of the tablets elaborated with sulphamethizole and HPMC/CD/lactose in the ratios shown in Table 1

Formulation	Experimental K_H (%·min$^{-0.5}$)	Predicted K_H (%·min$^{-0.5}$)	Formulation	Experimental K_H (%·min$^{-0.5}$)	Predicted K_H (%·min$^{-0.5}$)
1B-SUL	1.26 (0.07)	1.31	1H-SUL	1.26 (0.07)	1.26
2B-SUL	–	–	2H-SUL	–	
3B-SUL	–	–	3H-SUL	–	
4B-SUL	2.71 (0.18)	2.77	4H-SUL	3.07 (0.22)	3.07
5B-SUL	2.48 (0.19)	2.54	5H-SUL	2.59 (0.17)	2.58
6B-SUL	–	–	6H-SUL	–	
7B-SUL	3.97 (0.39)	4.14	7H-SUL	3.77 (0.34)	3.76
8B-SUL	2.37 (0.21)	2.16	8H-SUL	1.73 (0.11)	1.44
9B-SUL	5.25 (0.10)	5.19	9H-SUL	6.18 (0.42)	6.18
10B-SUL	5.28 (0.42)	5.23	10H-SUL	5.06 (0.52)	5.07

The results of the statistical analysis are shown in Fig. 4.

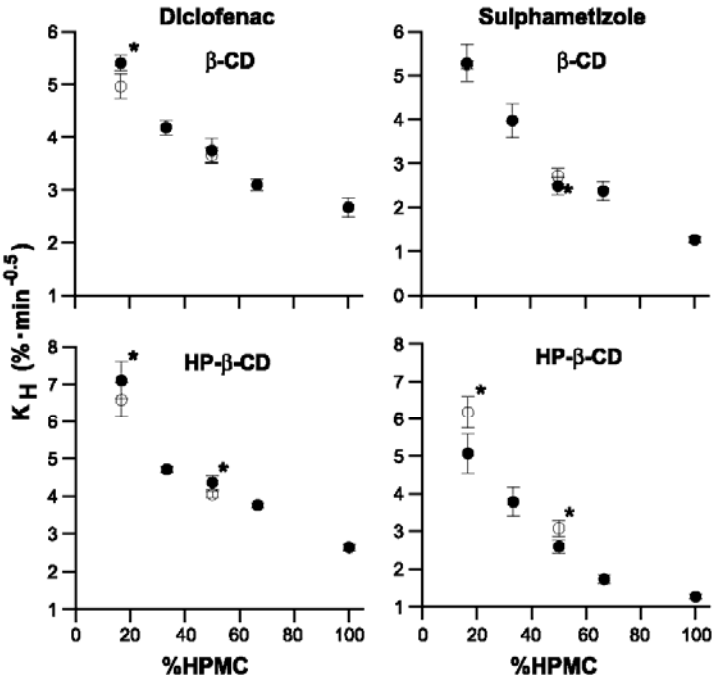

Fig 4. Effect of hydroxypropylmethylcellulose proportion on drug release rate from matrix tablets; for the formulations prepared incorporating CD and lactose in a different proportion (polymer percentage of 16.66% and 50%), those with a greater CD/lactose ratio (open symbols) showed lower diclofenac sodium release rates but greater sulphamethizole release rates (*significant differences at α level of 0.01).

nentially as observed for the diffusion coefficients through the gels, and as can be predicted by the hydrodynamic model developed by Phillies and Clomenil [31]. Nevertheless, the plots also highlight the effect of the presence of cyclodextrins compared to lactose. Interestingly, opposite effects were observed for diclofenac and sulphamethizole. For the most hydrophilic diclofenac, a higher cyclodextrin/lactose ratio significantly decreased the release rate (Table 4; Fig. 4). The concentration of polymer in the hydrated layer surrounding a HPMC matrix has been estimated by Lindner and Lippold [32] to be around 6%. Therefore, in the case of the matrix tablets prepared with mixtures of HPMC and cyclodextrin, the decrease in diffusivity of the drug incorporated to cyclodextrin complexes through the hydrated polymer layer should be even more evident than in the 2% HPMC gels. In contrast, formulations containing sulphamethizole showed an increase in drug release rate when the cyclodextrins are present in the matrix instead of lactose. Complexation with the cyclodextrins strongly increases sulphamethizole solubility in water [17], and, consequently, this enhances the release process. This effect was particularly important in the case of the most hydrophilic cyclodextrin, HP-β-CD. Rao et al. [11] also reported an increase in release rate of prednisolone from 25% HPMC K100M matrix tablets containing a highly water soluble cyclodextrin derivative, SBE-β-CD. Although the release tests are carried out using a volume of liquid much greater than the one needed to obtain a saturated solution of the drug, the release process initially involves the dissolution of the drug and, then, its diffusion through the gel layer of the hydrated tablet. During this process, cyclodextrins can act as solubilizing agents, promoting drug release, but also hinder the diffusion acting as obstacles in the diffusional path. The first effect will be dominant in the case of

hydrophobic drugs, and the second one in the case of hydrophilic drugs.

The second-order canonical equation derived from the simplex centroid design to which the formulation composition was adjusted is shown in Fig. 5. To calculate their coefficients, we used the rate constants obtained without considering the release profiles of formulations without polymer (nos. 2, 3 and 6), i.e., a

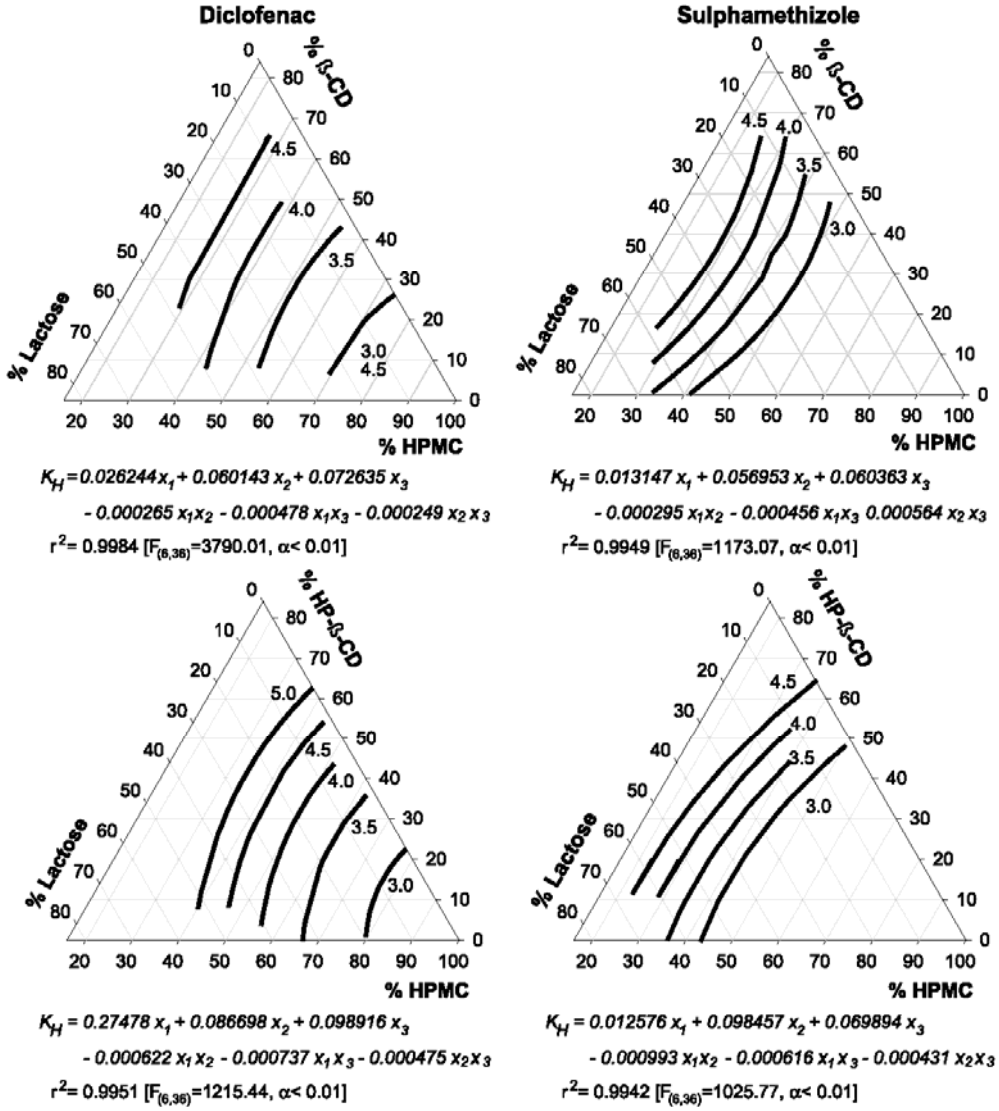

Fig 5. Contour plots of the release rate constants (K_H) estimated using the second-order canonical equations obtained by fitting to the experimental values shown in Tables 4 and 5 (x_1: % HPMC; x_2: % CD; x_3: % Lactose).

constrained model that do not take into account the formulations that have no acceptable mechanical properties and do not behave as matrices. The values predicted by the canonical equations fitted quite well the experimental values of K_H obtained for the formulations that contain, at least, 16.66% HPMC K4M, as can be seen in Tables 4 and 5 for values predicted with the second-order canonical equations. Since these equations had correlation coefficients, r^2, and F values as good as the third-order canonical equations, and the test points of the model, which are the data corresponding to formulation no. 7, provided values similar to the experimental ones, the second-order canonical equations should be selected to avoid the use of unnecessary complexes equations (Akaike Information Criterion) [25]. The values of the coefficients of x_2 and x_3 and the tendency shown in the contour plots clearly corroborate that, compared to lactose, cyclodextrins, especially HP-β-CD, delay the release of diclofenac and accelerate the release of sulphamethizol. The canonical equations are particularly useful to quantify the effect of the different variables and to predict to what extent the release profile can be modulated by choosing an adequate excipient mixture.

4. Conclusions

β-Cyclodextrin and hydroxypropyl-β-cyclodextrin strongly determine the diffusivity through gels and the dissolution rate from the tablets of drugs with which they can form inclusion complexes. For given HPMC and drug proportions, low CD concentrations promote drug diffusion by decreasing drug interactions with the polymer, while the opposite effect is observed for high CD concentrations; the free cyclodextrins increasing the tortuosity of the diffusional path. In the case of the tablets, an additional factor, the solubilizing capacity of cyclodextrins, needs to be considered. This effect is particularly important for the more hydrophobic sulphamethizol; the release rate being greater for HPMC matrix tablets containing cyclodextrins. In contrast, the delay in the release profile of the hydrophilic diclofenac may be attributed to the hindering effect of free cyclodextrins on drug diffusivity. The simplex centroid design used to prepare the tablets allowed to obtain the second-order canonical equations, which are particularly useful to predict the release behavior of tablets containing mixtures of HPMC, cyclodextrin and lactose.

Acknowledgements

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Sertaconazole-loaded cyclodextrin-polysaccharide hydrogels as antifungal devices.

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Sertaconazole-Loaded Cyclodextrin–Polysaccharide Hydrogels as Antifungal Devices[§]

Elena Lopez-Montero, Jose-Fernando Rosa dos Santos, Juan J. Torres-Labandeira, Angel Concheiro and Carmen Alvarez-Lorenzo[*]

Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain

Abstract: The aim of the present work was to develop novel hydrogels for delivering sertaconazole based on cyclodextrins and various biocompatible polysaccharides. Sertaconazole is an antifungal agent very effective for treatment of *Candida albicans* infections. However its poor aqueous solubility is still a challenging issue for developing suitable formulations. Complexation with cyclodextrins is a very attractive route to overcome this limitation, simultaneously enhancing its antifungal effectiveness. Hydroxypropyl-β-cyclodextrin (HPβCD) hydrogels prepared by direct cross-linking in presence of methylcellulose (MC), hydroxypropyl cellulose (HPC), hydroxypropyl methylcellulose (HPMC), carboxymethyl cellulose (CMCNa), or dextran were transparent and swelled in water without dissolving, which enables the formation of microenvironments very rich in cyclodextrin cavities responsible for hosting the drug and control its release rate. HPβCD hydrogels showed a high capability to load sertaconazole (with partition coefficients from 22 to 470) while still combining high water affinity (superabsorbency), versatile biomechanical properties (hardness and compressibility) and sustained release behavior (up to 4 days). Importantly, sertaconazol-loaded hydrogels showed effectiveness against *Candida albicans* in culture medium. HPβCD-polysaccharide hydrogels could be useful as sertaconazole delivery systems for the treatment of mucosal infections.

Keywords: Sertaconazole nitrate, cross-linked cyclodextrins, cellulose ether hydrogel, antifungal activity, *Candida sp.*

INTRODUCTION

Opportunistic fungal infections, particularly those caused by *Candida albicans*, are an important factor of mortality and morbidity in infants, children, and patients with compromised immune system [1-4]. Amphotericin B and azoles are currently the most used drugs to manage fungal infections [5]. Sertaconazole is an effective fungicidal and fungistatic agent and has a broad-spectrum activity against dermatophytes, opportunistic filamentous fungi, and also Gram-positive bacteria [6-8]. When used for the treatment of dermatologic and gynaecological infections, it presents a good profile of security, high cutaneous permanence and low systemic absorption [9]. Despite these valuable features, the extremely low aqueous solubility of sertaconazole (<0.01% w/v) strongly limits its practical use [10] and the search for an adequate delivery system is still a challenging issue. Complexation with β-cyclodextrin (βCD) and with hydroxypropyl-β-cyclodextrin (HPβCD) has been shown as an effective approach to enhance solubility and dissolution rate in aqueous medium [11-13]. Nevertheless, the relatively high stability constant of the sertaconazole complexes entails a high risk of drug precipitation after administration due to dilution of the complexes in the biological fluids, displacement of the equilibrium towards decomplexation, and release of the drug in the poor solvent medium [14]. Such a risk could be minimized by using cross-linked cyclodextrin hydrogels, which can swell in the biological medium without significant dilution [15, 16]. Hydrogels are outstanding patient-friendly delivery systems that enable a precise release of drugs at the desired site for a finite time. This enhances the bioavailability of the drug at the affected site of the organism with minimal systemic exposure and collateral effects [17, 18]. Current non-cyclodextrin hydrogels have been shown useful for the local treatment of dermal and mucosal infections, including *Candida albicans* [19-21]. However, their hydrophilic nature prevents an effective loading of hydrophobic drugs and usually leads to a rapid release of polar drugs [22]. In the particular case of antifungals with protonizable groups, loading has been promoted using hydrogels with oppositely charged groups, although the release rate resulted to be very dependent on drug solubility at the pH of the medium [23]. Recently, fluconazole-loaded acrylic hydrogels that showed swelling-controlled release behavior have been prepared [21]. An adequate combination of ionizable and non-ionizable monomers enabled to achieve different degrees and rates of swelling at vaginal pH and, consequently, different release rates. Cyclodextrin networks can offer novel features since they make use of an unique mechanism to control drug loading and delivery: the affinity of the drug for the cyclodextrin cavities [15, 16, 24-28]. Cyclodextrin hydrogels combine the ability of the cyclodextrins to host hydrophobic drugs with the viscoelastic behav-

*Address correspondence to this author at the Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782-Santiago de Compostela, Spain; Tel: +34-981563100 (14876); Fax: +34-981547148; E-mail: carmen.alvarez.lorenzo@usc.es

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ior and high water content of hydrophilic networks intended for topical or mucosal applications [15, 29].

In last years, we have developed a new approach to prepare in one step cyclodextrin hydrogels, with or without hydroxypropyl methylcellulose (HPMC), through condensation with ethyleneglycol diglycidylether (EGDE) [15, 30]. EGDE has two epoxy groups in its structure, both of similar reactivity and able to react with the hydroxyl groups of cyclodextrins and certain polysaccharides [31, 32]. This method does not require any modification in the cyclodextrin structure and takes places in aqueous medium under mild conditions, which are two important advantages towards an environmental-friendly ("green") chemistry. In previous papers, the contents in cyclodextrin and cross-linker and the kinetics of the cross-linking process were optimized, and the ability of the hydrogels to load and sustain the release of diclofenac and estradiol was demonstrated [15, 29, 33]. The aim of the present study was to develop and characterize novel hydrogels for delivering sertaconazole based on cyclodextrins and various polysaccharides (several non-ionic cellulose ethers, one ionic cellulose derivative, and dextran) of proved biocompatibility and used for first time for this application. The incidence of the nature and proportion of the polysaccharide on the swelling and mechanical properties of the hydrogels and on sertaconazole loading and release was studied in detail. Finally, the antifungal efficiency of drug-loaded hydrogels was tested in *Candida albicans* cultures.

MATERIALS AND METHODS

Materials

Sertaconazole nitrate (nitrate salt of 7-chloro-3-[-1-(2, 4-dichlorophenyl)-2-(1H-imidazol-1-yl)-ethoxy-methyl]benzo[b]thiophene; Mw 500.78 Da) was from Ferrer Internacional (Spain). Hydroxypropyl-β-cyclodextrin (HPβCD; D.S. 4.6, Mw 1310 Da) was supplied by Jansen Pharmaceutische (Belgium). Methylcellulose (MC, Methocel® A15C Premium EP, Mw 63, 000 Da) was from Colorcon Ltd. (UK); hydroxypropylcellulose (Nisso® HPC-M, Mw 570, 000 Da) from Nippon Soda Co. (Japan); hydroxypropyl methylcellulose (HPMC, Methocel® K4M, Mw 84, 200 Da) from Dow Stade GmbH (Germany). Sodium carboxymethylcellulose sodium (CMCNa, 400-800 cPs, Mw 125, 000 Da), sodium dodecylsulfate (SDS) and dextran from *Leuconostoc mesenteroides* (Mw 100, 000-200, 000 Da) were supplied by Sigma Aldrich (USA). Ethylenglycol diglycidylether (EGDE) was from Fluka Chemie GmbH (Germany). Water purified by reverse osmosis (MilliQ®, Millipore, Spain) with a resistivity above 18.2 MΩcm^{-1} was used. All other reagents were of analytical grade.

Phase Solubility Diagrams

Dissolutions of increasing concentration in HPβCD were prepared in water or in 0.25% w/v MC, HPC, HPMC, CMCNa or dextran aqueous solutions. Aliquots of these solutions (5 ml) were placed in ampoules containing sertaconazole in excess (20-25 mg). Some of these suspensions (two replicates) were autoclaved (Raypa AES-1219, Spain) at 121°C for 20 min. The autoclaved and non-autoclaved suspensions were shaken at 25°C and 50 rpm until equilibrium was reached (5 days), then filtered through 0.22 μm Millipore® cellulose acetate membrane filters (Teknokroma, Spain). The concentration of the dissolved drug was measured by UV spectrophotometry (Agilent 8453, Germany) at 302 nm. The apparent stability constant of the drug–cyclodextrin complexes was calculated from the slope (m) of the plot drug solubility (mM) versus HPβCD concentration (mM), and from the drug solubility in absence of cyclodextrins (S_o) [34].

$$K_{11} = \frac{m}{S_0 \times (1-m)} \quad (1)$$

Synthesis of HPβCD Hydrogels

Different amounts of MC, HPC, HPMC, CMCNa or dextran were added to 10 ml of HPβCD solution (20% w/w) in freshly prepared 0.2 M NaOH, up to a final concentration in polysaccharide of 0.4 or 0.8%. After homogenization, EGDE (4 ml) was added to each dispersion (10 ml) and stirred for two minutes at 20°C. The systems were immediately transferred to test tubes (10 mm internal diameter), which were hermetically closed and kept at 50°C for 24 h. After cooling down, the hydrogels were carefully removed from the moulds and immersed in water for 12 h to swell. Then, they were placed in a 10 mM HCl solution for 12 h to neutralize the alkaline medium and immersed in water once again. Finally, cylindrical pieces of each gel (4-5 mm thickness) were cut and maintained in water.

Characterization of HPβCD Hydrogels

Swelling

Dry samples of each hydrogel were immersed in 10 ml of water and weighed at pre-established time intervals. The kinetics of medium uptake was characterized by fitting the data obtained, up to 60% of the final content in water, to the following equation:

$$\frac{W_t - W_0}{W_\infty - W_0} = K_W \times t^{0.5} \quad (2)$$

where W_0 is the weight of the dried hydrogel, W_t the weight of the hydrogel at time t after immersion in the swelling medium, W_∞ the weight of the fully swollen hydrogel, and K_w is a rate constant. The equilibrium degree of swelling was estimated as follows:

$$Q = (W_\infty - W_0)/W_0 \quad (3)$$

Biomechanical Properties

Hardness and compressibility were determined using a TA-TX Plus Texture Analyzer (Stable MicroSystems Ltd., UK) fitted with a cylindrical aluminum probe (20 mm in diameter). A hydrogel disk of 8 mm thickness was placed on the platform and the probe was compressed into the sample at a defined rate of 1 mm/s and to a defined depth of 3 mm. Then, the probe was removed at 2 mm/s and the recovery of the sample was also monitored. Three replicate analysis of each sample were performed at room temperature. The hardness was estimated as the maximum resistance to compression (i.e. the peak value in the force-distance plot), and the compressibility was quantified as the work carried out in the compression (i.e. the area under the force-distance plot) [35]. The modulus of deformability, ED, was estimated from the initial linear portion of the force-distance plot, converting the force to a true stress using the expression:

$$\sigma_T = \frac{F(t)[h_0 - \Delta h]}{A_0 h_0} \quad (4)$$

and the distance to Hencky´s strain as follows:

$$\varepsilon_T = \ln\left(\frac{h_0}{h_0 - \Delta h}\right) \quad (5)$$

where h_0 is the original height of sample, Δh is the change in height, $F(t)$ the compressive force at time t, and A_0 the original cross-sectional area [36].

Sertaconazole Loading

Cylindrical pieces of each hydrogel (4-5 mm thickness) were placed in vials containing aqueous suspensions of sertaconazole (50 mg in 10 ml), which were put in a bath at 25°C and subjected to 50 oscillations per minute for one week; some being firstly autoclaved for 20 min at 121 °C. To determine the amount loaded, some hydrogels (three replicates) were immersed in 15 ml of 0.3% w/v SDS solution that were replaced every second day, for approximately one week, and the drug concentration in the washing medium was determined spectrophotometrically at 302 nm. The amount of drug loaded was estimated as the total amount of drug released to the washing medium. The amount loaded just by a simple equilibrium between the aqueous phase of the network and the loading solution was estimated using the following equation [37]:

$$\text{Loading (aqueous phase)} = (Vs/Wp)xC_0 \quad (6)$$

where Vs is the volume of water sorbed by the hydrogel, Wp the dried hydrogel weight, and C_0 the initial concentration of drug in the loading solution.

The affinity of the drug for the network was estimated as the partition coefficient, K_{NW}, between the polymeric networks and the drug loading solution, as follows [37]:

$$\text{Loading (total)} = [(Vs + K_{NW}Vp)/Wp]xC_0 \quad (7)$$

where Vp is the volume of dried polymer and the other symbols maintain the meaning of Eq. 6. The density of the dried hydrogels was assumed to be 1 g/ml.

Sertaconazole Release

Drug-loaded hydrogels were rinsed with water and immersed in 0.3% w/v SDS solution (30 ml, to ensure *sink* conditions) at room temperature. The drug concentration was measured spectrophotometrically in periodically taken samples and again placed in the same vessel, so that the liquid volume was kept constant. The experiments were carried out in triplicate. Once the test was finalized, the disks were weighed and they dried up to 50°C (Heraeus stove, Spain) until constant weight.

Antifungal Activity

The capability of sertaconazole to inhibit the growth of *Candida albicans* was analyzed in liquid YPD medium (peptone, yeast extract, and dextrose at 2% each). 0.75 ml of a fungal preinoculate in stationary phase of growth were added to YPD medium and the growth of *Candida albicans* was followed through the changes in absorbance at 600 nm. Sertaconazole-loaded hydrogels were placed in 15 ml of the *Candida albicans* culture in exponential phase of growth and the systems were maintained under stirring (180 rpm) at 30°C. The absorbance at 600 nm was periodically measured. The experiments were carried out in quadruplicate. Hydrogel disks without sertaconazole were used as controls. The percentage of growth was considered as the quotient of the absorbance registered for the medium to which sertaconazole-loaded hydrogels was added and for the medium containing *Candida albicans* without hydrogel.

RESULTS AND DISCUSSION

Dextran and four cellulose ethers with different substituents (MC, HPC, HPMC, and CMCNa) were chosen as components of HPβCD hydrogels due to their known hydrophilic character and biocompatibility [38, 39]. In addition, these polysaccharides alone have been shown to provide hydrogels with tuneable mechanical properties and potential as platforms for drug delivery [32, 40-42]. Complexation ability and, consequently, drug solubilizing efficiency are usually enhanced in the presence of hydrophilic polymers and by heating processes [34, 43]. Consequently, the effect of the chosen polysaccharides and of autoclaving on the complexation constant of sertaconazole with HPβCD was evaluated before preparing the hydrogels. The information obtained in this first step is relevant for understanding the loading/release behavior of the hydrogels [33].

Phase Solubility Diagrams

Sertaconazole solubility was notably enhanced in the presence of HPβCD, reaching up to 3 mM in 40 mM HPβCD (Fig. **1**). All diagrams were A_L type, which indicates the formation of 1:1 molar ratio complexes [34]. Table **1** shows the solubility values in solutions containing 1% (7.6 mM) HPβCD and 0.25% polysaccharide; 8-fold increase in sertaconazole solubility was observed in the HPβCD solution. The addition of CMCNa or MC slightly decreased the solubilization ability and the affinity constant, particularly when the HPβCD was above 10 mM and the systems were autoclaved. It is known that cellulose ethers can undergo precipitation or a sol to gel transitions when temperature rises; the polymer dehydrates and hydrophobic interactions among the cellulose backbones are promoted [39]. Such an increase in hydrophobicity can lead to a competition of the polymer with the drug for the cyclodextrin cavity, resulting in fewer cavities available for solubilizing sertaconazole.

Synthesis of HPβCD-Based Hydrogels

EGDE can act as a non-toxic cross-linker of cyclodextrins and polysaccharides forming ether bonds with hydroxyl groups; the reaction being catalyzed by OH^- ions and temperature [31-33]. We have observed that 0.2 M NaOH and 50 °C are suitable conditions for obtaining hydrogels without compromising the stability of the cyclodextrins and HPMC [15]. Thus, these conditions were maintained to prepare the novel HPβCD-polysaccharide hydrogels. Most glycidylether groups of EGDE are consumed in the reaction and, if any still remains in the hydrogel, the washing with 0.01M HCl aq. medium opens the rings to give hydroxyl groups [44].

The reactivity of the hydroxyl groups of HPβCD as well as those of cellulose ethers is greater for those at C2 and C6. In the case of dextran (α-D-1,6-glucose-linked glucan with

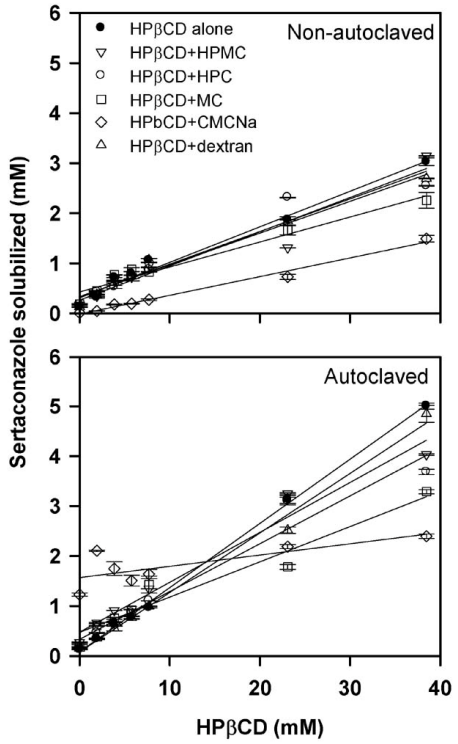

Fig. (1). Phase solubility diagrams of sertaconazole in aqueous solutions of HPβCD, in the presence or absence of 0.25% HPMC, HPC, MC, CMCNa or dextran.

Table 1. Sertaconazole Solubility in Media Prepared with 1% HPβCD and 0.25% of Various Polysaccharides, and Affinity Constants of Sertaconazole: HPβCD Complexes Before and After Being Autoclaved

System	Non-Autoclaved		Autoclaved	
	Solubility (mM)	K_{11} (M^{-1})	Solubility (mM)	K_{11} (M^{-1})
HP-βCD	1.06	610	0.96	1174
HP-βCD + 0.25% HPMC	0.97	587	1.35	887
HP-βCD + 0.25% MC	0.83	419	1.43	606
HP-βCD + 0.25% CMCNa	0.27	311	1.64	184
HP-βCD + 0.25% HPC	0.88	557	1.10	841
HP-βCD + 0.25% dextran	1.06	542	0.99	1082

side-chains 1-3 linked to the backbone units) the hydroxyls at C6 are occupied forming ether bonds among the glucose units to form the backbone, whereas some hydroxyls at C3 (around 5%) are substituted with branches of 1-2 glucose units long. The hydroxyl at C2 of the backbone as well as the hydroxyl at C2 and C6 of the branches can react with EGDE. The amount of EGDE added was sufficient to react with at least two thirds of all hydroxyls groups (of HPβCD and polysaccharide) present in the reaction medium. The polysaccharides were added to 20% HPβCD solutions up to 0.4 or 0.8%; the final concentration of polysaccharides in the pregel solution (after adding EGDE) being 0.29% or 0.57%, respectively. These concentrations were chosen to be below the critical entanglement concentration (i.e., ten-times the reciprocal value of the intrinsic viscosity reported in [45]) in order to obtain low viscosity solutions (before cross-linking) of individualized polysaccharide chains, avoiding inter- and intra-chain cross-linking and increasing the likelihood of an even distribution of both the polysaccharide and the HPβCD in the hydrogels. In fact, all hydrogels were viscoelastic and transparent and showed smooth and continuous surfaces.

Hydrogel Swelling

All hydrogels exhibited a fast swelling in the first 2 hours of the test, after which, the process slowed down until the equilibrium was reached approximately in 7 hours. Fig. (**2**) shows the swelling process of the HPβCD, HPβCD/MC and HPβCD/dextran hydrogels; the behavior of the other hydrogels being similar. Although the presence of the polysaccharide reduced the degree of swelling, all hydrogels took up high amounts of water and can be considered as superabsorbents (Table **2**). Hydrogels containing MC, CMCNa or HPC were the ones with the lowest degree of swelling, which can be attributed to the concomitance of two effects: i) a less hydrophilic character compared to HPβCD and ii) a higher degree of cross-linking due to an easier reaction of EGDE with the unsubstituted hydroxyl groups of cellulose.

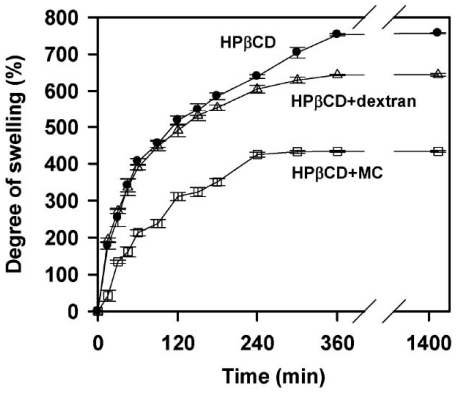

Fig. (2). Degree of swelling HPβCD hydrogels prepared without polysaccharides or with a 0.4% methylcellulose (MC) or dextran.

The swelling profiles fitted well to the square root kinetics, which implies that the water mainly penetrates in hydrogel by Fickian diffusion. The sorption rate constants (Table **2**) indicate that the water molecules can easily diffuse through the hydrogels.

Table 2. Degree of Swelling, Swelling Rate (r^2 >0.95), and Biomechanical Properties of HPβCD-Polysaccharide Hydrogels; Mean Values (Standard Deviations)

Hydrogel	Degree of Swelling (%)	Kw ($min^{-1/2}$)	Hardness (N)	Compressibility (N·mm)	Modulus of Deformability (ED; kPa)
HPβCD	765 (5)	0.048	4.22 (0.09)	3.1 (0.4)	35.5 (0.1)
HPβCD + HPMC 0.4%	710 (13)	0.048	4.29 (0.16)	3.9 (0.3)	43.8 (2.1)
HPβCD + HPMC 0.8%	664 (10)	0.050	5.87 (0.17)	5.0 (0.4)	57.0 (1.9)
HPβCD + MC 0.4%	433 (6)	0.061	9.33 (0.33)	7.8 (0.6)	115.0 (1.5)
HPβCD + MC 0.8%	514 (13)	0.062	3.31 (0.26)	2.5 (0.1)	49.5 (4.8)
HPβCD + CMCNa 0.4%	458 (13)	0.065	6.52 (0.36)	6.5 (0.8)	86.8 (1.9)
HPβCD + CMCNa 0.8%	456 (30)	0.071	9.38 (0.34)	8.2 (0.8)	110.0 (10.3)
HPβCD + HPC 0.4%	460 (6)	0.078	9.24 (0.78)	8.7 (0.5)	110.1 (10.3)
HPβCD + HPC 0.8%	417 (29)	0.071	5.82 (0.57)	4.2 (0.3)	89.1 (7.5)
HPβCD + Dextran 0.4%	644 (7)	0.049	3.97 (0.13)	4.3 (0.3)	45.5 (3.2)
HPβCD + Dextran 0.8%	568 (6)	0.048	4.7 (0.28)	3.7 (0.2)	59.3 (4.3)

Biomechanical Properties

Typical compression plots obtained for the water-swollen HPβCD hydrogels are shown in Fig. (**3**). In all systems, the force-distance curve registered during application of the force was almost superimposable to that obtained during the removal of the probe (i.e. recovery). The hardness and compressibility of hydrogels prepared with HPMC or dextran were similar to those of the HPβCD sole hydrogel (Table **2**) while the addition of other polysaccharides caused in general an increase in these parameters, which confirms the hypothesis of a greater effective cross-linking density in HPβCD/MC, HPβCD/CMCNa and HPβCD/HPC hydrogels. The HPβCD-based hydrogels are viscoelastic rather than purely elastic and, consequently, the Young's modulus cannot be strictly calculated from the slope of the force-distance plot [35, 36]. The cross-sectional area and length of the HPβCD-based hydrogel disks do change substantially while loads are applied, and the equations developed for extensional rheometry assuming incompressibility are not valid since the engineering stress ceases to be an accurate measure [46]. Therefore, the modulus of deformability, ED, was estimated using the Hencky model, in which the true stress represents an adjustment of the engineering stress $(F(t)/A_0)$ to account for cross-sectional area expansion of the deformed specimen [47]. ED is an index of the specimen stiffness and has been widely used for characterizing hydrogels and soft materials of varied nature [36]. It is interesting to note that hydrogels prepared with 0.4% MC, 0.4% HPC or 0.8% CMCNa were particularly stiff. In the case of these two non-ionic cellulose ethers, 0.4% caused an increase in the consistency of the hydrogels but 0.8% decreased again the consistency. This is due to that the presence of a moderate proportion of long cross-linkable chains increases the yield of the cross-linking between the cyclodextrins and cellulose chains and, consequently, a more rigid network is obtained. By contrast, greater proportions of long cellulose chains make the network able to deform to a great extent. This is because cyclodextrins become diluted among the cellulose chains and the likelihood of that EGDE reacts only with cellulose chains increases. Conformational changes of cross-linked cellulose chains are easier than in the case of the rigid HPβCD toroids, and thus relatively high proportions of MC or HPC enhance the capability of the hydrogels to deform under stress. Oppositely, the greater the content in CMCNa, the greater the stiffness of the hydrogels was. This finding should be related to electrostatic repulsions and osmotic effects caused by the ionic cellulose chains, which decrease the freedom of movement of the network. Thus, the incorporation of polysaccharide, even at low proportions, strongly determines the mechanical behavior of HPβCD-based hydrogels; the effect being very dependent on the structure and ionic nature of the polysaccharide chains. Nevertheless, all hydrogels evaluated showed ED values in the range of data previously found for other hydrogels and had the consistency required to be easily handled without risk of disintegration, but were also deformable enough to be mechanically biocompatible [29, 48].

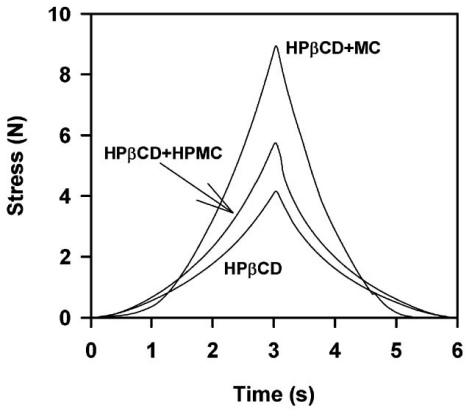

Fig. (3). Force-displacement curves for swollen HPβCD hydrogels prepared with 0.8% HPMC or 0.4% MC.

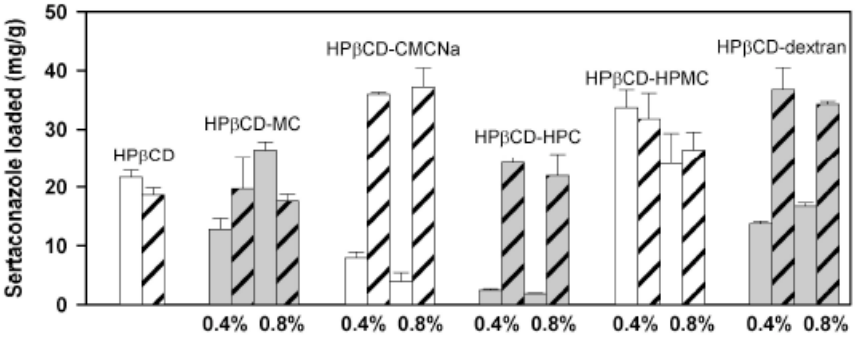

Fig. (4). Amounts of sertaconazole loaded by HPβCD hydrogels prepared without polysaccharides or with MC, CMCNa, HPC, HPMC or dextran at 0.4 or 0.8%. Columns with oblique-stripe fills identify hydrogels autoclaved during loading.

Loading and Release of Sertaconazole

Disks of each hydrogel were immersed in sertaconazole suspensions and some replicates were autoclaved to evaluate the effect of this thermal treatment on the loading capability. The hydrogels withstood this sterilization treatment without damage. Fig. (**4**) shows the amounts of sertaconazole loaded by each hydrogel, with or without applying autoclaving. HPβCD hydrogels loaded 21.7 mg/g and, when autoclaved, 18.7 mg/g. Hydrogels containing MC or HPMC loaded similar amounts or even greater. By contrast, non-autoclaved hydrogels made with HPC, CMCNa or dextran showed a significantly lower loading capability. Once autoclaved, HPC hydrogels reached similar values to those obtained for HPβCD sole hydrogels. In the case of CMCNa or dextran hydrogels, autoclaving enhanced so much the loading that these hydrogels become the ones with the greatest loading capability.

Sertaconazole can be loaded by diffusion into the inner aqueous phase of the hydrogel and by complexation with the cyclodextrin cavities. When the equilibrium is reached, the drug concentration should be the same in the aqueous phase of hydrogel as in the surrounding solution. Therefore, in the absence of other loading mechanisms, the greater the swelling of the hydrogels, the highest the loading in the aqueous phase [37]. Taking into account the aqueous solubility of sertaconazole (0.079 mg/ml), the maximum loading in the aqueous phase is 0.06-0.07 mg/g of hydrogel. These values are remarkably lower than any amount shown in Fig. (**4**). This means that most drug loaded by the network is interacting with its structural components, mainly HPβCD. In the case of HPβCD sole hydrogels, there are ca. 7 cyclodextrin units available per molecule of drug loaded, which means that the data shown in Fig. (**4**) are still far from saturation levels.

The affinity of the drug for the network was estimated as the partition coefficient, $K_{n/w}$, between the polymeric networks and the drug loading solution (Eq. 7) and resulted to be 273 and 235 for the HPβCD sole hydrogel before and after autoclaving. The HPβCD-polysaccharide hydrogels had $K_{n/w}$ values ranging from 22-30 for non-autoclaved HPβCD-HPC hydrogels to 454-470 for autoclave HPβCD-CMCNa hydrogels. This means that the hydrogels have a very remarkable affinity for the drug.

HPβCD-polysaccharide hydrogels have a 4% lower content in cyclodextrin than HPβCD sole hydrogels. This different content did not explain the notable differences observed in the amount of sertaconazole loaded by the different polysaccharide hydrogels. Hydrophobic sorption of drugs to MC and HPMC has been previously reported [15, 49]. Thus the hydrogels prepared with these polysaccharides loaded similar amounts to the HPβCD ones and showed similar $K_{n/w}$ values. On the other hand, CMCNa, HPC and dextran are more hydrophilic and this may create a barrier for the unspecific hydrophobic sorption of sertaconazole. Such a barrier can be overcome when autoclaving is applied, because of a temporal increase of the solubility of the drug in the aqueous medium that facilitates the complexation with the HPβCD units, which is the main driving force for the loading.

Sertaconazole release profiles from different hydrogels showed the influence of the polysaccharide used in their preparation as well as of autoclaving during the loading (Fig. **5**). All hydrogels showed a relatively fast delivery of drug in the first 24 hours, followed by a more sustained release step up to 4 days. It is important to note that the release studies were carried out under *sink* conditions in SDS micellar medium (as recommended for hydrophobic drugs) and, therefore, drug solubility is not a limiting step in the release profiles recorded. Sertaconazole powder dissolves in few minutes in SDS medium. Thus the sustained delivery is related to the capability of cyclodextrin cavities to retain the drug in the network, as previously reported for cyclodextrin-based hydrogels loaded with the hydrophobic hormone estradiol [33]. Decomplexation of a sertaconazole molecule from one cavity makes it available to complexate with a neighbour empty cavity; the likelihood of recomplexation being also dependent on the drug/cyclodextrin affinity. Therefore, drug movement through a hydrogel network dotted with many dimples can be envisioned as escaping a dimple to fall dawn in another one, which should be abandoned, and so on, up to reach hydrogel surface. The movement of a drug molecule may be faster when most dimples are occupied and the likelihood of recomplexation is less. Oppositely, as the hydrogel delivers the drug, the number of empty cyclodextrin cavities that are available for hosting the just-passing-through drug

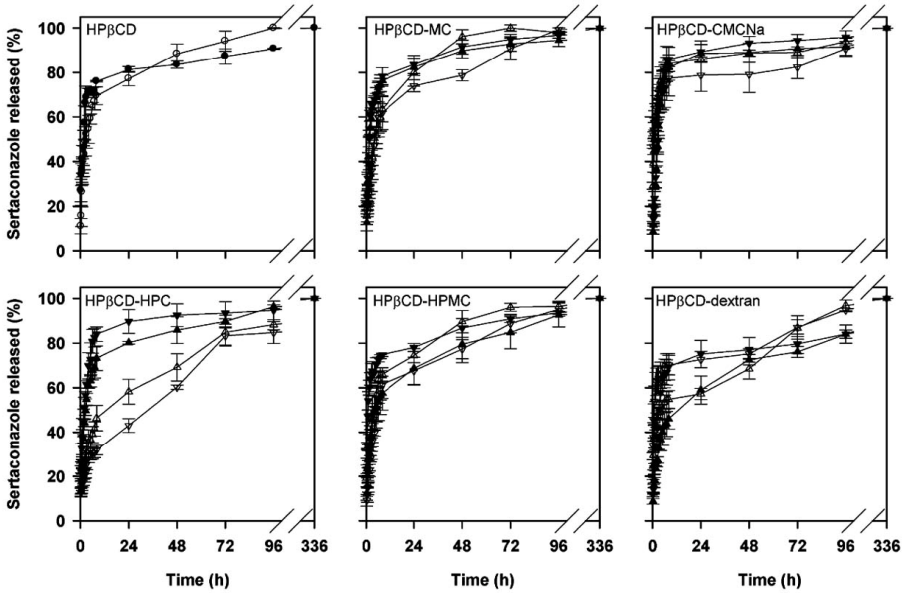

Fig. (5). Sertaconazole release profiles from HPβCD hydrogels prepared without polysaccharides or with MC, CMCNa, HPC, HPMC or dextran at 0.4% (up triangles) or 0.8% (down triangles). Full symbols correspond to hydrogels that were autoclaved during loading.

molecules increases. Furthermore, some previously released drug molecules could be attracted again towards the network. In the case of hydrogels containing polysaccharides, an increase in the cross-linking density (compared to HPβCD sole hydrogels) leads to an increase of tortuosity and to a minor mesh size which can also contribute to make drug release difficult. All these factors clearly explain that the non-autoclaved HPβCD/HPC hydrogels, which loaded the lower dose of drug, were those with the slowest release rate.

The amount of drug loaded by the HPβCD hydrogels, ca. 2%, is close to the content in drug of commercially available pharmaceutical creams, powders and solutions. As shown in Fig. (**5**), such content in sertaconazole is notably enhanced in HPβCD/HPMC hydrogels and in autoclaved HPβCD/CMCNa and HPβCD/dextran hydrogels. Taking into account the weight of the hydrogel disks prepared (~ 70 mg) and the antifungal activity of sertaconazole against *Candida albicans* (MIC_{50} = 0.07 mg/l; MIC = 0.63 mg/l), each HPβCD hydrogel disk contains sertaconazole enough to decrease the population of fungi to the half if immersed in 20 litres of medium, and to kill all fungi in a volume of 2 litres. Thus, sertaconazole-loaded disks when enter in contact with the small physiological volumes at the common sites of *Candida sp.* infections (e.g. vagina or mouth) should be adequate to efficiently treat this type of infections. The versatility of the hydrogels is increased by the fact that the size of the disks can be fixed at will to adjust the dose to specific requirements.

Antifungal Activity

The antifungal effectiveness of the sertaconazole-loaded hydrogels was verified using *Candida albicans* cultures in exponential phase of growth. Sertaconazole inhibits the ergosterol synthesis and, consequently, alters the cellular membrane formation causing the killing of the fungi [7]. Hydrogels without sertaconazole were used as controls in order to evaluate the direct effect of the cross-linked networks on the growth of *Candida albicans*. As can be observed in Fig. (**6**, dark grey columns), the networks themselves only caused a minor decrease in the growth rate. As a positive control, the same amount of drug (40 mg) as the maximum dose loaded by the hydrogel disks was dispersed in the culture medium (white column with horizontal lines, in Fig. (**6**)). Such a suspension diminished the *Candida* population up to a 50% in 24h. Sertaconazole-loaded hydrogels also significantly decreased the growth rate of *Candida albicans*. The inhibitory effect was particularly relevant at 24 h for hydrogels prepared with HPβCD sole or combined with MC or CMCNa, which killed the fungi as efficiently as the drug suspension despite of being loaded with lower amount of sertaconazole. Despite the culture medium is not as good solvent as the SDS micellar solutions used for the release experiments, hydrogels prepared with HPβCD sole or combined with MC or CMCNa are expected to deliver the drug faster than the others. Those hydrogels that sustained more the release, mainly prepared with HPC or HPMC, required more time to begin to evidence the antifungal effect.

CONCLUSIONS

Versatile cyclodextrin-polysaccharide hydrogels with tuneable biomechanical properties and capability to load sertaconazole and to regulate its release rate were obtained in a single-step using EGDE as cross-linker. The nature and proportion of the polysaccharide components play an impor-

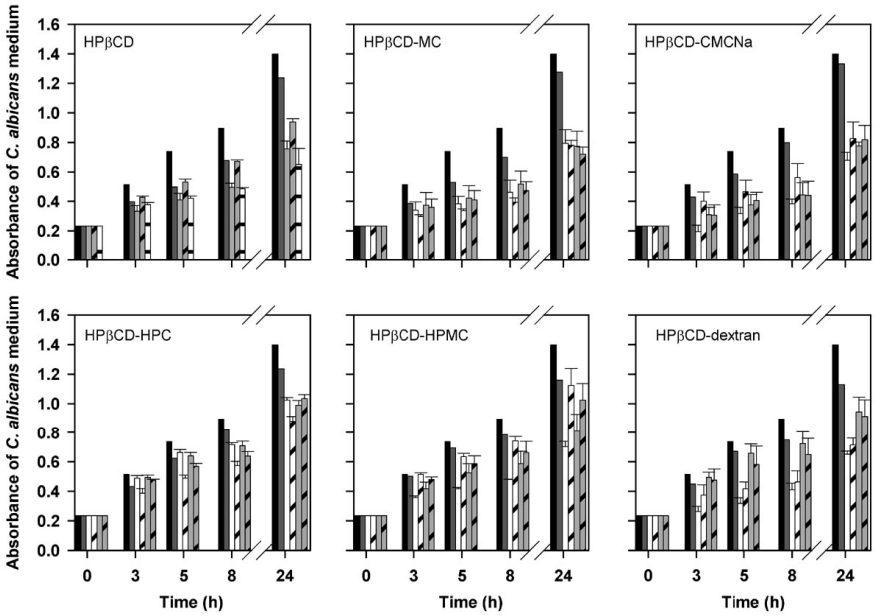

Fig. (6). *Candida albicans* growth in control medium (black column), in the presence of non-loaded hydrogel (dark grey column), or in the presence of sertaconazole loaded hydrogels. Upper left plot shows the effect of sertaconazole-loaded HPβCD hydrogels prepared without polysaccharides (grey columns) and of a drug suspension prepared with the maximum dose loaded by the hydrogels (white column with horizontal lines). The rest of the plots show the effect of sertaconazole-loaded HPβCD hydrogels prepared with different proportions of polysaccharides (0.4% white columns or 0.8% grey columns). Columns with oblique-stripe fills identify hydrogels autoclaved during loading.

tant role in the performance of the hydrogels. Sertaconazole loading is mainly driven by the drug affinity for cyclodextrin units. Autoclaving is also revealed as a key factor to facilitates the complexation and thus to regulate the delivery of the drug release. These hydrogels are capable of loading therapeutic doses of sertaconazole, to control its release for more than 24 hours and to provide proper antifungal activity against *Candida albicans*. The beginning of action was faster for those hydrogels that loaded more drug and released it faster. Therefore, the new cyclodextrin-polysaccharide hydrogels have a great potential as efficient carriers of antifungal drugs to be applied topically or on mucosa.

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Influence of culture conditions of *Gordonia jacobaea* MV-26 on canthaxanthin production

Patricia Veiga-Crespo[1]
Lucía Blasco[2]
Fernando Rosa dos Santos[3]
Margarita Poza[1]
Tomás G. Villa[1,2*]

[1]Department of Microbiology, Faculty of Pharmacy, University of Santiago de Compostela, Spain
[2]School of Biotechnology, University of Santiago de Compostela, Spain
[3]Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela, Spain

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*Corresponding author:
T.G. Villa
Department of Microbiology
Faculty of Pharmacy, Campus Sur
15782 Santiago de Compostela, Spain
Tel.: +34-981563100 ext.14949.
Fax: +34-981592490
E-mail: mpvilla@usc.es

Summary. Commercial interest in the use of natural pigments isolated from microorganisms has increased in recent years; hence, molecules belonging to the polyisoprenoid group (i.e. β-carotene, astaxanthin, and canthaxanthin) have been the focus of much attention. The bacterium *Gordonia jacobaea* readily synthesizes and accumulates large amounts of canthaxanthin (β-β′-carotene-4,4′-dione), which is widely used in the food and cosmetics industries. In the present work, the effects of different low-cost raw materials on fermentation and canthaxanthin accumulation by a hyperpigmented strain of *G. jacobaea* were studied. Canthaxanthin production and peak levels of accumulation varied according to the different media used. [**Int Microbiol** 2005; 8(1):55-58]

Key words: *Gordonia jacobaea* · canthaxanthin · carotenoids · soy-meal · fermentation

Introduction

Canthaxanthin (β-β′-carotene-4,4′-dione) is a ubiquitous keto-carotene that is of considerable industrial interest because of its widespread use in both the food and cosmetic industries [12]. While synthetic forms of canthaxanthin and many other pigments are currently available, their use in the food industry has been questioned by consumer agencies, so that the development of new sources of natural pigments has become essential to meet the increasing demand.

Due to their ease of manipulation, microorganisms provide an excellent system for the large-scale production of carotenoids, as has been shown with the yeast *Phaffia rhodozyma* [13] and the bacterium *Brevibacterium* KY-4313 [14], which accumulate the natural carotenoids astaxanthin and canthaxanthin, respectively. However, the inability of these two sources to meet world-wide demand has spurred research aimed at finding new sources (Table 1) and at optimizing fermentation technologies.

Gordonia jacobaea MV-1 (gram-positive, catalase negative, G + C 61%), which was isolated in this laboratory during a routine screening of pigmented microorganisms, is able to accumulate several carotenoids, including the keto-carotenoid *trans*-canthaxanthin [8]. However, its low carotenoid content (200 μg/g dry weight) does not support its industrial application. After several rounds of mutations, a hyperpigmented mutant (MV-26) with enhanced canthaxanthin and β-carotene accumulation was obtained. This mutant accumulates six-fold more canthaxanthin than the wild-type strain [9].

The influence of growth conditions and medium composition on the carotenoid synthesis pathway has been reported previously. This pathway begins with isoprenyl pyrophosphate (IPP), which may be formed either from mevalonic acid (MVA) or through the glyceraldehyde phosphate/pyruvate pathway [4,11]. Thus, media rich in these or related pre-

Table 1. Microbial sources of carotenoids

Microorganism	Main carotenoid	Yield	Reference
Haematococcus pluvialis	Astaxanthin	1.30 mg/l	[16]
Phaffia rhodozyma	Astaxanthin	30 µg/g	[13]
Halobacterium salinarium	Astaxanthin	265 µg/g	[7]
Dictyococcus cinnabarinus	Canthaxanthin	1 mg/g	[14]
Brevibacterium KY-4313	Canthaxanthin	2 mg/l	[14]
Haloferax alexandrinus	Canthaxanthin	2156.67 µg/g	[2]
Muriellopsis sp.	Lutein	22.7 mg/g	[10]
Blakeslea trispora	Lycopene	40 mg/l	[6]
Flavobacterium sp.	Zeaxanthin	0.09 µg/l	[1]
Dunaliella salina	ß-Carotene	2.12 mg/l	[15]
Dunaliella bardawil	ß-Carotene	20.1 pg/cell	[3]

cursors can be used to increase the carotenoid yield in industrial fermentations. In the present study, different media and low-cost raw materials were assayed in order to determine the optimal conditions of canthaxanthin production by *G. jacobaea* MV-26.

Materials and methods

Strains and culture conditions. The strain employed was the hyperpigmented *Gordonia jacobaea* MV-26 [9]. The bacterium was grown in different commercial media: yeast extract peptone dextrose (YPD) (20 g peptone/l, 10 g yeast extract/l, 20 g glucose/l), tryptone soy-meal broth (TSB) (3 g soy-meal peptone/l, 2.5 g glucose/l, 17 g casein peptone/l, 5 g dipotassium hydrogen phosphate/l, 5 g NaCl/l) and brain heart infusion broth (BHIB) (12.5 g calf-brain infusion solids/l, 5 g beef-heart infusion solids/l, 10 g protease peptone/l, 2 g glucose/l, 5 g NaCl/l, 2.5 g di-sodium phosphate/l). Low cost media consisting of different proportions of soy meal (0.5%–2%), beet molasses (0.5%–5%), and a mixture of soy meal (0%–5%) and glucose (0%–10%) were also assayed.

G. jacobaea MV-26 was grown in 1-l flasks containing 250 ml of the appropriate medium at 30°C or 37°C in a rotary shaker (150 rpm) for 8 days. In the case of YPD medium, screening was implemented for 10 days. Every other day, aliquots were withdrawn, and the levels of β-carotene and canthaxanthin (Fig. 1) were evaluated. After this first screening, *G. jacobaea* was grown in 2 l of each medium: BHIB, TSB, YPD, 0.5% soy meal, and 1.5% glucose/2% soy meal at 30°C in a rotary shaker (150 rpm) since these media resulted in the highest levels of canthaxanthin production. When necessary, *G. jacobaea* was plated on medium supplemented with agar (2%).

Growth curve of *Gordonia jacobaea* MV-26. Growth curves were established either by determining the optical density at 600 nm in a Beckman DU-40 spectrophotometer or by plate counting (colony-forming units, CFU) when complex media were used (i.e. soy-meal-based).

Analysis of pigment production. Samples (3 ml) were withdrawn every 24 h and 1 ml of 0.1 M potassium phosphate buffer (pH 7) and 3 ml hexane (Merck) were added. After vigorous vortexing, the samples were centrifuged for 10 min at 5000 rpm to allow phase formation. The organic phase was filtered through a 0.22-µm filter (Gelman Sciences) and the pigment content was evaluated. When pigment production was analyzed in 2-liter cultures, an additional ethanol extraction was performed. In this case, following maximum canthaxanthin production, the biomass was harvested at 4°C by means of continuous-flow centrifugation at 15000×g. The pigments were subsequently extracted by resuspending the cells in pure ethanol.

Carotenoid pigments were analyzed by HPLC using a silica-gel column (Teknokroma, 5-µm pore size, 25-cm length and 45-mm diameter). The mobile phase was hexane/ethyl acetate (1:1 v/v) (Romil). The flow was 1 ml/min and the pressure was 0.4 kpsi; the injection volume was 30 µl and the temperature was 25°C. HPLC analysis of carotenoid pigments extracted with ethanol was carried out by adding one volume of hexane plus 1 ml 0.1 M potassium phosphate buffer (pH 7). After centrifugation for 10 min at 5000 rpm, the hexane phase was recovered and filtered as described previously. The peaks were evaluated based on their absorption at 480 nm. Retention times and concentrations of the samples were compared with pure standards of β-carotene and canthaxanthin.

Statistical analysis. The results of the influence of the growth media on canthaxanthin production were subjected to statistical analysis with the SPSS 12.0 program.

Results and Discussion

G. jacobaea MV-26 grew in all of the media, except those containing only beet molasses. While in all media supporting growth the production of carotenoids varied, maximum canthaxanthin accumulation consistently occurred in the stationary phase of growth (Fig. 2). Moreover, pigment production in *G. jacobaea* followed a pattern in which peak canthaxanthin production was inversely correlated with peak β-carotene production. Thus, as observed in other organisms [5], the keto-carotene canthaxanthin is the end-product of the carotenoid pathway, and β-carotene is an intermediate.

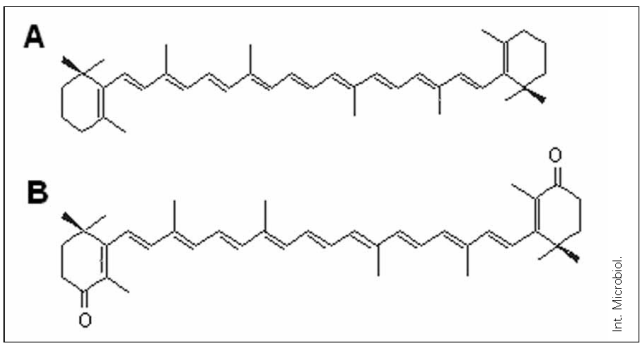

Fig. 1. β-Carotene (**A**) and canthaxanthin (**B**) molecules.

Standard calibration curves prepared with known amounts of pure β-carotene and *trans*-canthaxanthin allowed quantification of these pigments in the different media (Table 2). The highest levels of canthaxanthin were produced in soy-meal-based media, especially when supplemented with glucose as carbon source. In a previous study [8], an effect of the carbon source on carotenoid synthesis and accumulation was observed. Among all the carbon sources tested, glucose induced the highest level of canthaxanthin synthesis, probably by favoring the overproduction of mevalonic acid, which is a key metabolite in the synthesis of polyprenoid-derived carotenoids. Accordingly, beet molasses should have been a good medium for canthaxanthin production. Thus, the inability of *G. jacobaea* to grow on molasses-based media was most likely due to the presence of an inhibitor in the industrial raw material.

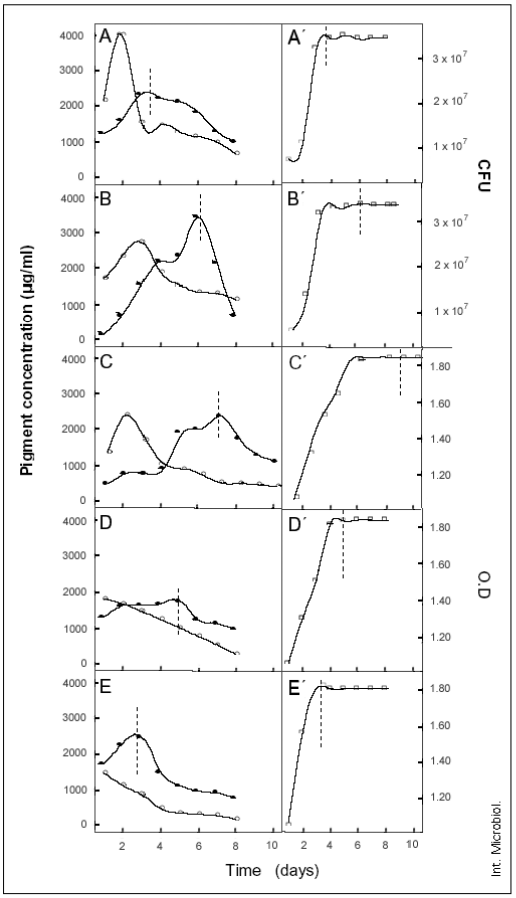

Fig. 2. Concentrations of canthaxanthin (µg/ml) (•) and ß-carotene (µg/ml) (○) produced by *Gordonia jacobaea* MV-26 in different production media: (A) 0.5% soy meal/0% glucose; (B) 2% soy meal/1.5% glucose; (C) YPD; (D) TSB; (E) BHIB. (A´-E´) represent the evolution of growth of *G. jacobaea* (□) in the different media. The dashed vertical line marks the peak of production and the bacterial growth phase.

The production profile varied both in quantity and timing depending on the medium used. Thus, in BHIB media the maximum pigment concentration (2600 µg/ml) was measured on day 3 of fermentation (stationary phase), whereas in TSB medium the maximum (1500 µg/ml) was attained on day 6 (stationary phase). In the case of YPD medium, a maximum yield was obtained on day 9 (stationary phase), but it was always lower than the yield from BHIB medium. When soy-meal-containing media were compared, 5% soy meal/1% glucose was found to elicit a peak of around 13000 µg/ml, while 2% soy meal/1.5% glucose afforded a peak of nearly 3500 µg/ml. However, despite the higher level of canthaxanthin production, the use of 5% soy meal/1% glucose medium was ruled out because of the difficulties involved in handling this mixture. Instead, it was concluded that the addition of 0.5% soy meal/0% glucose could be implemented in order to increase canthaxanthin production. The ability of soy meal to increase pigment production was most likely due to the presence of precursors of the carotenoid pathway, such as mevalonic acid or related substances, which, together with the extra amounts of glucose present in the media, could have increased canthaxanthin production. These putative precursors might also have been responsible for the increases in β-carotene and canthaxanthin that occurred during the first few days of the stationary phase of growth, an effect that was particularly observable using BHIB medium, which is rich in terpenoid precursors, this leading to the emergence of an earlier peak of pigment production.

Varying the temperature from 30°C to 37°C had little effect on canthaxanthin production (data not shown). However, a larger range of temperatures should be tested before an effect of temperature on canthaxanthin synthesis can be completely ruled out.

Statistical analysis, based on Student's *t*-test (95% confidence interval), confirmed the observations on the differential effects of the tested media on pigment production by *G. jacobaea* MV-26.

Table 2. Yields of canthaxanthin production by *Gordonia jacobea* MV-26 in different production media

Medium	Canthaxanthin (µg/ml)
YPD	2140
BHIB	2489
TSB	1760
10% glucose/0% soy	1800
0% glucose/0.5% soy	2340
5% glucose/1% soy	1000
2% glucose/1.5% soy	2650
1.5% glucose/2% soy	3440
1% glucose/5% soy	13373

A comparison of the abilities of hexane and ethanol to extract canthaxanthin showed that the extraction capacity of hexane was 50% higher than that of ethanol. However, since hexane is not allowed in foods, ethanol extraction must be optimized for *G.. jacobaea*. Nonetheless, ethanol extraction of carotenoids from this bacterium is an advantage compared to other carotenoid-producing microorganisms, such as *Phaffia rhodozyma*, in which this solvent is ineffective unless the yeast has previously been disrupted. Moreover, the use of ethanol in pigment extraction lowers the possibility of toxicity and animal intoxication due to contamination with residual organic solvents in downstream processes.

Taking into account that higher concentrations of soy meal hampered fermentations because of its insolubility, the medium containing 0.5% of soy meal was chosen as the optimum to obtain the highest and the more profitable pigment production. The production profiles in higher volumes were similar to those observed for lower volumes. In general, soy meal alone, even without glucose added, promoted pigmentation and the amount of carotenoids was also higher.

The current widespread concern about the use of genetically modified organisms (GMO) in processes related to the food industry makes classical approaches, such as the one described here, of commercial relevance as the best and safest way for increasing the concentrations of important biomolecules.

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Influencia de las condiciones de cultivo de *Gordonia jacobea* MV-26 en la producción de cantaxantina

Resumen. El interés comercial del uso de pigmentos naturales aislados a partir de microorganismos se ha incrementado en los últimos años y las moléculas pertenecientes al grupo de los poliisoprenoides (p.e. β-caroteno, astaxantina y cantaxantina) se han convertido en un foco de atención. La bacteria *Gordonia jacobaea* es capaz de sintetizar y acumular grandes cantidades de cantaxantina (β-β´-caroteno-4,4´-diona), muy usada en la industria alimentaria y de cosméticos. En este trabajo estudiamos la influencia de diferentes materias primas de bajo coste en la fermentación y la acumulación de cantaxantina por una cepa mutante hiperpigmentada de *G. jacobaea*. Se ha observado que la producción de cantaxantina y el momento en el que se alcanza la máxima producción varía según los diferentes medios empleados. [**Int Microbiol** 2005; 8(1):55-58]

Palabras clave: *Gordonia jacobaea* · cantaxantina · carotenoides · medio de soja · fermentación

Influencia das condições de cultivo de *Gordonia jacobaea* MV-26 na produção de cantaxantina

Resumo. O interesse comercial do uso pela indústria alimentar de pigmentos de origem natural isolados a partir de microorganismos experimentou um aumento consideravel nos últimos anos, em especial aqueles com uma estructura carotenóide (β-caroteno, astaxantina e cantaxantina) A bacteria *Gordonia jacobaea* presenta uma grande capacidade para produzir e acumular grandes quantidades de cantaxantina (β-β´-carotene-4,4´-dione), muito usada pela indústria alimentar e cosmética. O presente trabalho utiliza cepas hiperpigmentadas de *Gordonia jacobaea* modificadas geneticamente para analizar a influencia de diferentes matérias primas de baixo custo, na fermentação e acumulação de cantaxantina. A produção de cantaxantina e o momento em que esta é máxima variou consoante o meio de cultivo utilizado. [**Int Microbiol** 2005; 8(1):55-58]

Palavras chave: *Gordonia jacobaea* · cantaxantina · carotenóides · meio de cultivo de soja · fermentação

Bioremediation of Polycyclic Aromatic Hydrocarbons in Marine Environments.

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Bioremediation of Polycyclic Aromatic Hydrocarbons in Marine Environments

Trinidad de Miguel*, Lucía Feijoo-Siota, Fernando Rosa dos Santos and Tomás G. Villa.
Department of Microbiology and Parasitology. Faculty of Pharmacy. University of Santiago de Compostela. 15782 Santiago de Compostela. Spain

Abstract

Marine pollution by petroleum crude oil arising from human activities is currently one of the most concerning environmental problems. The composition of crude is a mixture of substances, among them polycyclic aromatic hydrocarbons (PAHs). The importance of removing PAHs from the environment is rooted in their mutagenic and carcinogenic properties as well as in their toxicity. The use of microorganisms to solve environmental problems is known as bioremediation. Bioremediation techniques have gained importance in the last few decades owing to their many advantages over the physico-chemical and mechanical methods used for such purposes. The aim of the present chapter is to present an updated overview of the toxicity of PAHs, the use of natural and genetically engineered microorganisms for their degradation, the bacterial metabolism of PAHs and the parameters involved in the rate of biodegradation.

Correspondence/Reprint request: Dr. Trinidad de Miguel. Department of Microbiology and Parasitology. Faculty of Pharmacy. University of Santiago de Compostela. 15782-Santiago de Compostela. Spain. E-mail: mptrini@usc.es

1. Introduction

Over the past few decades the introduction into the environment of synthetic (xenobiotics) or natural compounds at a much higher rate than should usually occur in nature has led to important environmental problems. One of the main sources of pollution of terrestrial and maritime media is the huge amount of crude oil released into the sea. This contamination may result from releases from manufacturing and refining installations, spillage during transportation and discharge works, accidents, and the sinking of oil tankers, or from deliberate releases such as the one that occurred in Kuwait in 1991 during the Gulf War [1, 2, 3].

Maritime catastrophes such as the *Prestige* or the *Exxon Valdez* incidents are always a huge concern to public opinion due to the serious problems caused to the ecosystems of the relatively small areas affected [4, 5]. Despite their enormous impact on the environment, they only contribute to a small extent to the total amount of crude oil released into the sea [6, 7], which has been estimated at between 1.7 and 8.8 metric tons annually [8].

The ability of certain microorganisms to use hydrocarbons as the sole sources of carbon and energy was reviewed for the first time in 1946 [9, 10]. Since then, large oil spills, which have occurred worldwide [2], have led the scientific community to study the use of microorganisms to solve the problems

of contamination deriving from such accidents. The use of microorganisms to solve environmental problems is known as bioremediation and bioremediation techniques have gained importance in recent decades due to the fact that they are the only processes that ensure complete mineralization of toxic compounds, and hence their removal [11].

2. The composition of crude oil

The chemical composition of crude is a complex mixture of hydrocarbons with other possible elements, such as heavy metals [12]. Petroleum hydrocarbons can be divided into four classes: i) saturated or aliphatic hydrocarbons (n-alkanes, branched alkanes and cyclic alkanes), ii) mono- or polycyclic aromatic hydrocarbons, iii) asphaltenes (phenols, fatty acids, ketones, esters and porphyrines), and iv) resins (pyridines, quinolines, carbazoles, sulphoxides and amides) [13].

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic compounds consisting of two or more fused benzene rings in linear, angular or clustered arrangements [14]. Many of them are acutely toxic or genotoxic [15] and all of them persist for long periods of time when widespread in the environment [16]. Usually, a higher number of benzene rings decreases the solubility and increases the toxicity of these compounds.

3. Toxicity of PAHs

The toxicity of PAHs was reported for the first time in 1761, when the physician John Hill recognized the link between the use of snuff and nasal cancer [17]. Many PAHs have toxic, mutagenic and/or carcinogenic properties [18, 19]. Their liposolubility means that they are readily absorbed by the gastrointestinal tracts of fish and mammals, mainly accumulating in fat tissues. Thus, they are incorporated to trophic chains and are potential causative agents of serious health problems or genetic defects in humans. The marine animals affected by oil spills show different concentrations of PAHs accumulated in their fat tissues. This is mainly due to the duration of their life cycle and to their different detoxification abilities. The higher the life expectancy of a given species, the higher the accumulation of contaminants through the diet, and life expectancy is usually directly related to the organism's size. Hence, in general larger size means more bioaccumulation of toxins. Another factor to be considered is the specific detoxification ability of different species. Thus, bivalve mollusks, which have limited or no detoxification capacity, show high concentrations of PAHs accumulated in their fat tissues. In contrast, fish have effective detoxification mechanisms, resulting in much lower accumulations of toxins. It is worth noting that a higher detoxification ability is linked to greater toxicity for the organism owing to the compounds resulting from PAH

metabolization via the cytochrome P450 oxidase system. This is why high mortality and carcinogenic effects are observed in fish but are negligible in bivalves. Since humans have a high detoxification capacity, PAHs are extremely toxic for our organism [12].

The US Environmental Protection Agency (US EPA) has identified 16 PAHs on the basis of their abundance and toxicity as priority pollutants [19] (fig. 1).

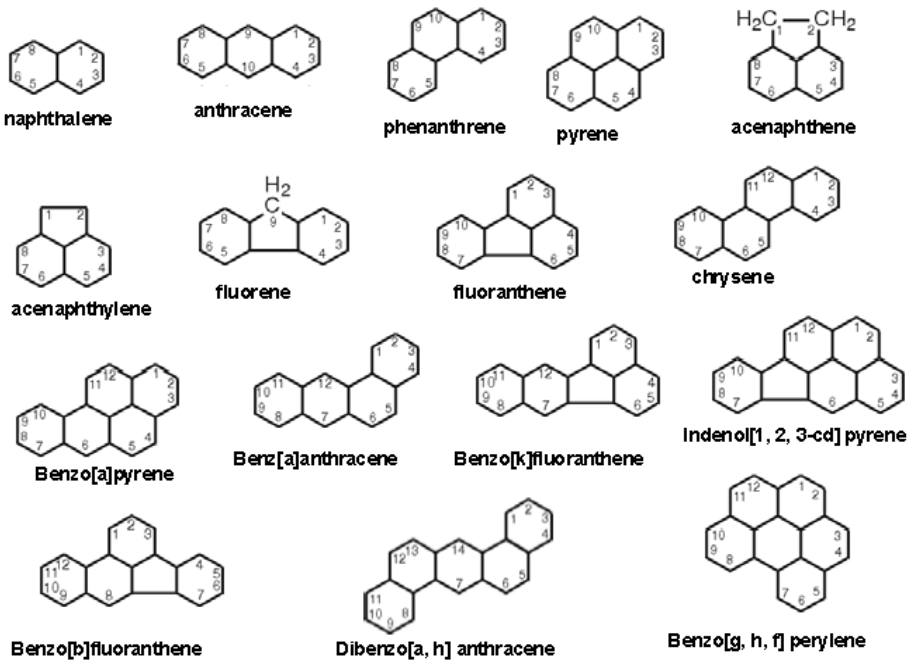

Fig. 1. Chemical structures of 16 priority PAH compounds identified by the US EPA

Benzo(a)pyrene is the most toxic PAH for humans. Thus, it is used to standardize the chemical composition of crude hydrocarbons in terms of toxicity. In this sense, equivalences between the toxicity of benzo(a)pyrene and that of other hydrocarbons are established and the total toxicity of crude is estimated in equivalents of that compound [12].

4. Bioremediation of PAHs

Owing to their extreme toxicity, persistence and abundance in sea water and along the coast, it is clear that the elimination of PAHs from polluted environments is of great importance.

Different technologies have been proposed or used to clean oil-contaminated environments. The biological treatment of soil and water polluted with PAHs is a reasonably inexpensive and versatile alternative to physicochemical and mechanical treatments, and it offers the advantages of low cost, good safety and little environmental disturbance [14]. Biological technologies include the use of straw or plant material as absorbents for crude [2], biosurfactants to clean oiled surfaces [20], biological polymers coating surfaces to avoid oil adhesion [2], and bioremediation using microorganisms. Microorganisms enable organic pollutants be completely mineralized to inorganic materials (CO_2, H_2O, NO_3^-, etc), which are then integrated into natural biogeochemical cycles [21], whereas other techniques such as soil washing, simply move the contaminants from one location to another [22]. Nevertheless, there are many factors which may affect the course of a successful bioremediation process. Besides the physical conditions and the nature of the pollutant hydrocarbons present, the concentration and decontamination rates of the different classes of hydrocarbons, substrate bioavailability and the properties of the biological system involved all play important roles in the feasibility of using bioremediation to solve a contamination problem [23].

An accurate definition of bioremediation might be “the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes” [24]. This definition includes the addition of both degrading microorganisms and other substances that stimulate the action of such microorganisms. Accordingly, two types of bioremediation could be considered: biostimulation, consisting of the addition of both nutrients and electron acceptors to the environment in order to stimulate indigenous microbial populations, and bioaugmentation or seeding, consisting of the introduction of exogenous microbial populations able to degrade the pollutants [14].

PAH-degrading microorganisms can be isolated from PAH-contaminated soils and open water [17, 25, 26]. If the actual presence of degrading microorganisms can be assumed, the availability of nutrients (especially nitrogen and phosphorous) is regarded as the most common limiting factor [27]. Some investigators have used inorganic and organic fertilizers to stimulate the action of microorganisms in open-water and shoreline field trials [2]. Such fertilizers contain nutrients, such as phosphate, nitrogen and potassium, which are necessary for the growth of degrading microorganisms, frequently included in oil matrices to prevent the formation of emulsions and reduce oil viscosity and interfacial tension. The results obtained from these field trials show that

biostimulation could be a potential method for cleaning oil-contaminated shorelines, but that it is of no use in open-water [2].

Several studies have been carried out on the effects of seeding on contaminated shorelines and soil. These include PAH-degrading consortia or pure strains [28, 29, 30]. However, the efficiency of this technique for bioremediation in large-scale experiments has not been demonstrated [16]. This is due to the inability of exogenous species to co-exist with endogenous flora in colonizing the environment. Selective pressure on the bacterial genome exerted by laboratory cultures may elicit a lack of protective and adhesive cell structures, rendering the cell unable to survive in certain natural environments where adhesion and protection are of great importance [31]. Johnsen et al. [16] reported that bioaugmentation should not only consist of the addition of a metabolic function but that it should "influence the bioavailability of pollutants when the application methods involve homogenization, slurrying, or intensive flushing of the system, or when the bacteria added differ from the indigenous population with respect to their specific affinity for the contaminant, maintenance requirements, ability to co-utilize natural substrates, active or passive mobility, adhesion behavior, or ability to produce biosurfactants and to ingest surfactant-solubilized chemicals".

Actually, most of the positive results obtained from *in situ* studies using bioaugmentation are seen a few weeks after seeding. When the experiments are carried over longer periods of time, biodegradation rates decrease as the exogenous microorganisms disappear [32, 33]. In this sense, only the results obtained from seeding experiments carried out in fermentors or chemostats seem to be clearly positive [27]. In these cases, competition with the autochthonous flora is reduced or absent and the biodegradation parameters can be adjusted to achieve the highest rates [34, 35, 36].

5. PAH-degrading microorganisms

A large number of PAH-degrading microorganisms has been isolated and tested for effective mineralization of naphthalene and anthracene (two and three aromatic rings) [37]. These include species of the genera *Alcaligenes*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Corynebacterium*, *Bacillus*, *Moraxella*, *Streptomyces*, *Vibrio* and *Cyclotrophicus* [38, 39, 40]. The degradation of four-ring PAHs (fluoranthene, pyrene, chrysene and benz[a]anthracene) has also been documented [26, 38]. Strains metabolizing four-ring PAHs belong to the genera *Stenotrophomonas*, *Mycobacterium*, *Gordonia*, *Rhodococcus*, *Pseudomonas*, *Burkholderia*, *Flavobacterium* and *Cycloclasticus* [26, 41, 42, 43]. Notwithstanding, microorganisms capable of utilizing PAHs with more than four rings (e.g., benzo[a]pyrene) as sole carbon and energy sources have not been found [23]. Despite this, the mineralization of these compounds has been well characterized in studies addressing co-metabolic

transformations [44, 45, 46, 47, 48, 26]. Co-metabolism consists of the degradation of PAHs without the generation of energy for cellular metabolism. It takes place as the result of a non-specific enzymatic reaction, with a substrate competing with the structurally similar primary substrate for the enzyme's active site [16]. In pure cultures, co-metabolism is a dead-end transformation without benefit to the organism, whereas in a mixed culture or in the environment such an initial co-metabolic transformation may pave the way for subsequent attack by another organism [49].

6. Factors affecting PAH-bioremediation

The most successful examples of bioremediation have been reported in studies carried out under controlled conditions at laboratory scale or in limited natural areas where the monitoring of certain parameters is relatively easy. Nevertheless, the applicability of bioremediation technologies in natural environments is subjected to external factors, most of which are unpredictable and difficult to control.

6.1. Temperature

Temperature has a considerable effect on the success of *in situ* bioremediation. Some of the positive results obtained at laboratory scale using bioaugmentation or biostimulation are difficult to reproduce under real conditions; i.e., in natural areas where the land or the water temperature is subjected to seasonal changes.

Temperature affects bioremediation because of its influence in the physical nature and chemical composition of the oil and in the choice of the microbial population to be used [9]. High temperatures increase the solubility of PAHs, this increasing their bioavailability and biodegradation [50]. High temperatures also reduce oil viscosity and increase the volatilization of short-chain alkanes [27], this promoting biodegradation. On the other hand, oxygen solubility is negatively affected by increasing temperatures, which reduce the metabolic activity of aerobic microorganisms [51].

In general, degradation rates are seen to decrease with decreasing temperatures [27], probably due to the "Q_{10}" effect on the enzymes involved [52].

Most PAH-degrading microorganisms operate at mesophilic temperatures, although the biodegradation of crude oil has been reported at temperatures as low as 0ºC in sea water [50] and –1.1ºC in soil at [53]. In contrast, thermophilic enzymes from ligninolytic fungi harnessed for the degradation of PAHs have been reported to operate at temperatures over 75ºC [54].

6.2. Oxygen

Until recently, it was believed that aerobic conditions were necessary for the degradation of hydrocarbons in the environment [27]. Although evidence had appeared for the anaerobic biodegradation of petroleum hydrocarbons [55, 56, 57], its ecological significance was generally considered to be low [5, 9, 58, 59].

In recent years, however, increasing evidence has emerged of anaerobic PAH-degradation with nitrate and sulfate as electron acceptors [16, 51]. The anaerobic biodegradation of naphthalene, phenanthrene, anthracene and pyrene has been assayed using pure cultures of *Pseudomonas* sp. and *Vibrio* sp. [60, 61, 62]. Several *in situ* studies carried out in anaerobic environments suggest that the potential for anaerobic PAH-biodegradation may be greater than previously recognized [63, 64, 65].

6. 3. Nutrients

The high concentrations of hydrocarbons present in areas affected by oil spills often produce excessive carbon/nitrogen or carbon/phosphorous ratios, which are unfavorable for microbial growth [27]. As mentioned above, it is therefore a common practice to supplement contaminated sites with fertilizers (usually containing nitrogen and phosphates) with a view to stimulating the endogenous microbial community, thus enhancing bioremediation [2]. Although many authors have confirmed the effectiveness of such fertilizer use [27], few (and then controversial) studies have explored the optimal C:N:P ratio for the degradation of PAHs [66, 67, 68]. Further work in this area aimed at determining the most favorable nutrient levels for the optimal degradation of PAHs would certainly benefit the success of bioremediation [51].

6.4. pH

Most PAH-degrading microorganisms operate at pH values close to neutrality [27]. This is an important factor to be considered when bioremediation is to be applied on soils, whereas in open water its influence is negligible due to the very small variations in pH that occur in such an environment, even when challenged by large oil spills.

Several studies have reported that extreme pH values decrease the activity of hydrocarbon-degrading microbial populations [69, 70]. Since the pH of polluted sites is often linked to the pollutant itself, the indigenous flora may not be able to transform PAHs under such new pH conditions [51]. A common practice in soil-bioremediation is therefore to adjust the pH by the addition of lime [71].

There are several reports on PAH-bioremediation at extreme pH values. Stapleton et al. [72] described the degradation of naphthalene, phenanthrene and anthracene in an acidic soil (pH 2) by a consortium of fungi and bacteria.

Recent research with different *Pseudomonas* species isolated from alkalophilic environments has revealed the ability of such species to degrade naphthalene in liquid culture at elevated pH [51]. Some of the isolates were able to reduce the pH of the liquid media from 9 to 6.5 within 24 hours.

6.5. Bioavailability

The term bioavailability can be defined as the effect of physico-chemical and microbiological factors on the rate and extent of biodegradation [51]. PAHs are hydrophobic organic contaminants with low water solubility that are resistant to biological, chemical and photolytic breakdown [73]. This means that they have low bioavailability. The larger the molecule of PAH, the lower its solubility and hence its bioavailability [74].

Some attempts to increase the bioavailability of PAHs for the purpose of bioremediation have assayed surfactants. This is of special interest in the case of the bioremediation of contaminated soils, since PAH molecules tend to adsorb to mineral surfaces making the pollutant harder to extract [51].Surfactants, or detergents, are compounds that contain both a hydrophobic and a hydrophilic moiety, thus providing a "bridge" between the hydrophobic PAH molecule and the hydrophilic microbial cell [51]. Some bacteria produce such substances as a strategy for increasing PAH transfer. In this case, they are called biosurfactants. Biosurfactant production is not very common among PAH-degraders, and obviously not essential for PAH degradation, but does help the microorganism to increase the bioavailability of certain compounds [16].

6.6. Toxicity of end-products

When designing a bioremediation trial it should be taken into account that the success of any bioremediation method involves not only the removal of the contaminants but must also prevent the formation of more toxic breakdown metabolites [51]. A recent study on PAH-bioremediation has reported the formation in a bioreactor of metabolites such as PAH-ketones, quinones and coumarins, which are more toxic and persistent than the actual parent compounds [75]. Some eco-toxicological tests have been developed and used to ensure toxicity reduction after bioremediation trials [76, 77].

7. PAH-metabolism

There are basically three different mechanisms for the aerobic metabolism of PAHs: bacterial, ligninolytic fungal, and non-ligninolytic fungal (fig. 2). All of them are based on the oxidation of the aromatic ring, followed by systematic breakdown of the compound to PAH metabolites or mineralization to carbon dioxide. As seen above, anaerobic metabolism is also possible via the

hydrogenation of the aromatic ring following PAH degradation with nitrate and sulfate as electron acceptors [51].

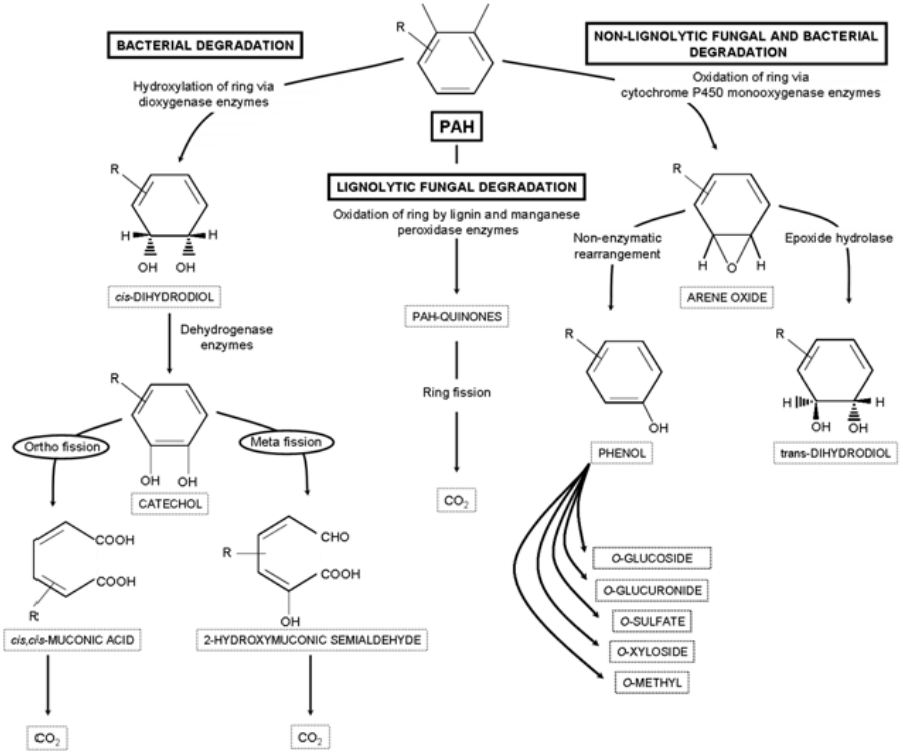

Fig. 2. Schematic representation of the three different mechanisms of the aerobic degradation of PAHs by microorganisms.

8. Bioremediation using genetically engineered microorganisms (GEMs)

Recently several GEMs have been successfully constructed for bioremediation purposes [78]. Microbial genes have been tailored or reshuffled to obtain strains with new biodegradation capabilities. These include the creation of new metabolic routes; expansion of the diversity and substrate ranges of already existing pathways; enhancement of xenobiotic bioavailability, and the optimization of biocatalysts [79].

Nevertheless, the general tendency in bioremediation has been the use of natural microorganisms rather than genetically manipulated ones [22]. This is due not only to ethical issues and adverse public reaction, but mainly to regulatory constraints concerning the release of GEMs into the environment, since there is no fail-safe system to guarantee successful containment of the GEM released after completion of the bioremediation process [80,81]. In principle, GEMs are usually less competent than their progenitors for survival in wild environments, and hence their release should not be an environmental problem of concern. Despites this, there are some reasons for being careful when releasing GEMs into the environment. First of all, there are noteworthy examples of genetic manipulations that have enhanced the competitive fitness of microorganisms [82]. In addition, although it has been proposed that the genetic manipulation of organisms would produce crippled individuals unable to survive in the wild, certain hazards that may arise after their release, including the elimination of the maladaptative effects of some genes through evolution, can have serious environmental consequences [82, 83]. The horizontal exchange of genes among bacteria in the environment is another factor that can hinder the use of GEMs for bioremediation purposes. Many studies have demonstrated such genetic interactions in nature [84, 85, 86, 87, 88, 89, 90, 91]. Peters et al. [85] reported the horizontal transfer of genes of selective value as long as 6 years after the introduction of a microorganism for the bioremediation of phenol at a polluted site.

Assuming that society can be convinced that GEMs are beneficial and that they can contribute to the quality of human life, it is necessary to establish certain conditions for a GEM before its release into the environment in order to minimize all possible risks. In this regard, it would be desirable that the design of the microorganism should ensure that it would be viable only under the specific conditions of its intended use (e.g., in the presence of a contaminant), or that it would incorporate self-destruction mechanisms that could be induced when a certain environmental condition arises [92]. One bio-containment strategy is the also known as the suicide system. This involves “suicide genes”, which are induced in the absence of pollutant, eliminating the GEM from the population when the bioremediation process has been completed, or when cells escape from the polluted environment [93, 94, 95]. In order to avoid the problem of horizontal gene transfer, “suicide vectors” containing conditionally lethal genes have been constructed, such that the genes cannot survive transfer to another microorganism [92]. Although, many biologically contained microorganisms have been constructed to date [22], only the genetically manipulated strain of *Pseudomonas fluorescens* designated HK44 has been released into a contaminated soil environment for bioremediation purposes [96, 97]. *Ps. fluorescens* HK44 contains the naphthalene catabolic plasmid pUTK21 and a transposon-based bioluminescence-producing *lux* gene fused

within a promoter for the naphthalene catabolic genes [98]. Thus, this strain degrades naphthalene and at the same time produces a detectable bioluminescent signal.

In spite of the foregoing, self-containment genetic systems are not completely safe. Accordingly, the current potential of GEMs in bioremediation should be restricted to emergency situations that cannot be managed properly using other technologies [96].

9. Conclusions

Over the past few decades, maritime catastrophes have caused serious damage to coastal ecosystems worldwide. Environmental contamination with PAHs can be alleviated through the use of different bioremediation techniques, some of which have provided good results at laboratory scale or at confined sites. Until now, *in situ* bioremediation techniques cannot compete with physico-chemical or mechanical methods, although a number of advantages with respect to these procedures make bioremediation a promising technology that demands further study and investment.

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Biodegradation of Naphthalene by *Pseudomonas stutzeri* in Marine Environments: Testing Cells Entrapment in Calcium Alginate for Use in Water Detoxification

L. Feijoo-Siota,
F. Rosa-Dos-Santos,
T. de Miguel, and
T. G. Villa
Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Santiago de Compostela, Spain

ABSTRACT The biodegradation of naphthalene in sea water by freely suspended and alginate-entrapped cells of *Pseudomonas stutzeri* 19SMN4 has been investigated in batch cultures. The results showed that immobilized cells can be stored at 4°C for 1 month without loss of viability. The biodegradation was highly affected by the availability of nitrogen and phosphorous, so at 30°C a naphthalene concentration of 25 mM was almost completely degraded (93%) by free cells in 6 days in samples supplemented with these nutrients, whereas only 42% naphthalene was consumed in the nonsupplemented samples. Biodegradation was much slower at 16°C than at 30°C; after 6 days of culture at 30°C, almost all naphthalene was degradated by free and immobilized cells, whereas only 22% and 34% at 16°C, respectively. The degradation rate remained unaffected when the naphthalene concentration was reduced from 25 to 10 mM. Alginate of three different viscosities was used for immobilization of cells. After 7 days of culture, beads formed with 31.4 cP alginate were fragmented, whereas beads formed with 240 and 3600 cP did not display structural changes and afforded the same degradation rate. Beads formed with high-viscosity alginate retained cells more efficiently.

KEYWORDS alginate, immobilization, naphthalene, *Pseudomonas*, seawater

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are considered as important environmental pollutants (Soniassy et al., 1994). The marine environment is subjected to contamination by PAHs from a variety of sources, mainly of anthropogenic nature (García et al., 1998), as well as by large-scale oil spills. In quantitative terms, crude oil is one of the most important sources of contamination and it has been estimated that such pollution represents between 1.7 and 8.8 $\times$ 10^6 tons of petroleum hydrocarbons that impact marine

Address correpondence to Trinidad de Miguel, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Santiago de Compostela, Campus Sur, 15782 Santiago de Compostela, Spain.

waters and estuaries annually (Head and Swannell, 1999).

Naphthalene is a PAH that has been classified as a potential human carcinogen by international agencies (the International Agency for Research on Cancer [IARC], the US Environmental Protection Agency [US EPA], and the Deutsche Forschungsgemeinschaft [DFG]) (Preuss et al., 2003). Naphthalene is among the most toxic components in the water-soluble fraction of crude oils and has been shown to be concentrated in vertebrate and invertebrate marine organisms (Sharanagouda and Karegoudar, 2001). Its toxicity, together with its chemical persistence, means that this compound is extremely dangerous as an environmental contaminant (Bamforth and Singleton, 2005).

Biological methods of treatment have proved to offer good alternatives for the degradation of naphthalene. Several reports on the naphthalene degradation by different microorganisms have appeared (Manohar and Karegoudar, 1998; Abou Seoud and Maachi, 2003), but there are very few reports addressing marine environments.

The release of hydrocarbons into aquatic environments that contain low concentrations of inorganic nutrients often produces unfavorable carbon/nitrogen or carbon/phosphorous ratios for the microbial growth (Van Hamme et al., 2003). The lack of availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbons in seawater (Leahy and Colwell, 1990). Temperature is another important factor affecting the biodegradation of PAHs. Increasing temperatures facilitate the solubility of PAHs and hence their bioavailability. Degradation rates are generally observed to decrease with decreasing temperature; this is believed to be a result of diminished rates of enzymatic activity and the low bioavailability of PAHs at low temperatures (Bamforth and Singleton, 2005).

The species of the genus *Pseudomonas* are metabolically versatile. They are able to use a large number of organic compounds, among them aromatic hydrocarbons, as their sole carbon and energy sources (Palleroni, 1984). Within this genus, the species *Pseudomonas stutzeri* 19SMN4 is able to take advantage of naphthalene as the sole carbon and energy source. This trait is conferred by plasmid pLIB119, which codifies naphthalene degradation pathways (Rosselló et al., 1991).

The immobilization of hydrocarbons degraders may solve the problem of the dilution of microorganisms in open waters. In comparison with free cells, the use of immobilized cells has several advantages: (i) a high population density can be achieved in a limited volume; (ii) it limits substrate inhibition and toxicity to microorganisms by diffusional constrains; (iii) it affords cellular protection against adverse environmental conditions; (iv) it is a reusable application, which reduces overall costs and facilitates cell storage over long periods with no loss of their degrading capacities (Quek et al., 2006).

The degradation of naphthalene using immobilized *Pseudomonas* spp. in several different matrices has been studied previously (Manohar and Karegoudar, 1998; Manohar et al., 2001; Abou Seoud and Maachi, 2003). Thus, K-carrageenan, alginate, agar, polyacrylamide-hydrazide, and polyurethane foam have been used successfully for the immobilization of microorganisms. Alginate has several advantages over others. In addition to its high porosity and chemical stability, it offers a mild, fast, simple, and cheap immobilization method (Fukui and Tanaka, 1982). The stability of alginate spheres and the prevention of their breakdown are important factors for the proper long-term functioning of immobilized beads. However, there are several variables that affect the mechanical strength and durability of alginate beads, among them viscosity (Darrabie et al., 2006).

The aim of the present study is to quantify the degradation of different initial concentrations of naphthalene by free and immobilized *Pseudomonas stutzeri* 19SMN4 cells in conditions similar to the marine environment, with a view to estimating the effects of different viscosities of calcium alginate on cell release and degradation, and testing the appropriateness of supplementing the medium with mineral nutrients.

MATERIALS AND METHODS

Bacterial Strain and Culture Media

The naphthalene-degrading microorganism *Pseudomonas stutzeri* 19SMN4 used in this study was isolated from marine sediment enrichment with 2-methylnaphthalene (Rosselló et al., 1991).

The medium used for preculture of the bacterium contained (per liter): 12.8 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.246 g of $MgSO_4 \cdot 7H_2O$, and 0.147 g of $CaCl_2 \cdot 2H_2O$. The pH of the medium was adjusted to 7 and the medium was sterilized by autoclaving at 121°C for 15 min. The

sterilized medium was then supplemented with 15 mg L^{-1} of Tween 80 and naphthalene (0.1% *w/v*) (Fluka; purity >99%). Preculture of the bacterium was carried out at 30°C on a rotary shaker at 180 rpm.

For naphthalene degradation studies, seawater collected from a beach on the Galician coast (Lagos beach, Spain), (pH 8.20, and salinity 33 mg L^{-1}) was used. When necessary, the seawater was supplemented with K_2HPO_4 as a source of phosphorous at a C/P ratio of 30:1, and NH_4NO_3 as a nitrogen source, at a C/N ratio of 10:1 (Ahn et al., 1998). Naphthalene was added to the sterilized recipients at a final concentration of 25 mM or 10 mM from stock solutions in hexane (80 g L^{-1}). After evaporation of the solvent, the culture medium was added to the naphthalene and subjected to ultrasonic treatment for 5 min.

The samples were incubated in the darkness to avoid the photooxidation of naphthalene (McConkey et al., 2002).

Analytical Method

Naphthalene was extracted from spent medium with an equal volume of hexane (Panreac; PAI) (Aranha and Brown, 1981). The mixture was vortexed for 5 min in order to dissolve the naphthalene in the organic phase. The mixture was then transferred to a Corex tube and centrifuged at 12000 rpm for 5 min in a Beckman J2-MC centrifuge in order to separate the organic and aqueous phases. A 1-ml aliquot was taken from the organic phase and naphthalene was quantified by HPLC (Waters), the injection volume being 10 μl. Detection was carried using a Waters 996 Photodiode array detector (wavelength 254 nm). A standard curve ($y = 0.0133x$, $R^2 = .9994$; x = naphthalene concentration [mM], y = HPLC peaks area) was constructed from different naphthalene concentrations in hexane (1, 5, 10, 15, 20, 25, and 30 mM), each measurement being carried out in triplicate.

Immobilization Method

Alginate entrapment of cells was performed according to the method of Bettman and Rehm (1984), with modifications.

The cells from an early stationary-phase preculture of *P. stutzeri* were routinely harvested by centrifugation and washed twice in a 0.9% NaCl solution.

Three different alginic acid types were used: Aldrich 180947 (viscosity 31.40 cP) and Sigma A0682 (low viscosity 240 cP) and A2033 (medium viscosity 3600 cP).

Alginate was dissolved in 0.9% NaCl overnight and sterilized by autoclaving (121°C, 15 min). Alginate beads containing immobilized *P. stutzeri* cells were prepared as follows: 1 g of alginate was dissolved in 40 ml of 0.9% NaCl overnight and sterilized by autoclaving; 2 g of *P. stutzeri* cells (wet weight) were resuspended in 8 ml of sterile 0.9% NaCl and added to the previous solution, stirring to complete homogenization.

The mixture was extruded drop-wise through a hypodermic needle (0.9 × 25; 20 G) into a cold, sterile solution of $CaCl_2$(0.2 M) and 0.9% NaCl (Sigma-Aldrich). The beads were left to harden in the same solution at room temperature and gentle stirring for 1 h. Finally, the beads were washed several times with 0.9% NaCl to remove excess calcium ions and untrapped cells.

The beads had an average diameter of 1.6 mm and were stored at 4°C in propylene tubes containing 2 g of beads each. Sterile beads (without microorganisms) at the different viscosities were used to monitor the abiotic loss of naphthalene.

Batch Cultures

Batch cultures were performed using 125-ml flasks with 10 ml seawater and 1 ml culture at the end of the log phase or 2 g of alginate beads containing immobilized *P. stutzeri* cells.

The cultures were supplemented with naphthalene (10 or 25 mM), as indicated above, and with nitrogen and phosphorous when necessary.

Uninoculated flasks and flasks with sterile alginate beads were used as negative controls in order to evaluate the abiotic loss of naphthalene due to evaporation.

All flasks were incubated at 30°C or 16°C on a rotary shaker at 150 rpm. At preset intervals, samples were removed and the entire content was used to determine the degradation of naphthalene. The samples were stored at −20°C and processed together at the end of the experiment. All experiments were performed in duplicate.

Enumeration of Viable Immobilized Cells

Previously weighed alginate beads were rinsed twice with a sterile 0.9% NaCl solution and suspended in 10 ml of a sterile 1% solution of sodium citrate (Panreac) (pH 6) (Klinkenberg et al., 2001). The suspension was vortexed to achieve a complete dissolution

of the alginate and spread on Luria broth (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) after serial dilution.

The colonies were counted on agar plates after incubation for 48 h at 30°C. All counts were performed in duplicate.

RESULTS AND DISCUSSION

In this study, the effects of different factors affecting the biodegradation of naphthalene in seawater were investigated. The results on biodegradation are depicted as the ratio of the residual and initial concentration (C/C_0) against time.

The experiments with sterile alginate beads were used to evaluate the abiotic loss of naphthalene. Using this parameter, the results obtained were corrected in order to prevent the abiotic naphthalene loss due to evaporation or adsorption to the beads from being attributed to bacterial degradation.

Effect of Storage on the Viability of Immobilized Cells of *Pseudomonas stutzeri* 19SMN4

Immobilized cells were stored at 4°C and −20°C in tubes containing 2 g of alginate beads. Cell viability was analysed after 15 and 33 days, as described above. The counts of colony-forming units (CFU) in fresh-made beads and after 15 and 33 days of storage at 4°C and −20°C are shown in Table 1. The results show that the immobilized cells can be stored at 4°C for at least 1 month with no appreciable loss of viability, whereas storage at −20°C is not as suitable. The same study was carried with all three types of alginate beads, and the same results were observed (results not shown). This is advantageous for practical use of the beads, because it shows that they do not require any special or expensive storage conditions.

TABLE 1 Effect of Storage at 4°C and −20°C on the Viability of Immobilized Cells (Alginate Viscosity 31.4 cP)

	Viability (CFU/2 g)	
Time (days)	4°C	−20°C
1	3.0×10^9	
15	1.5×10^9	1.2×10^6
33	1.9×10^9	4.8×10^5

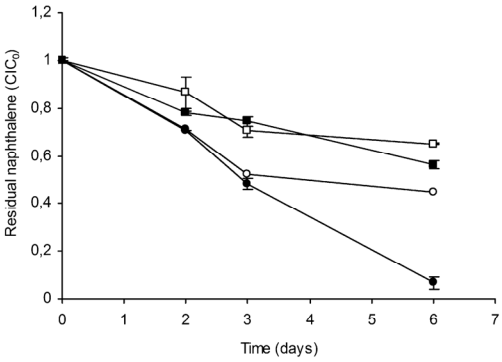

FIGURE 1 Biodegradation of naphthalene by free cells in seawater at 30°C with an initial concentration of naphthalene of 25 mM with (—●—) and without (—■—) nutrients; and at 16°C, with an initial concentration of 10 mM with (—○—) and without (—□—) nutrients. Error bars represent the *SD* of duplicate replicates.

Effect of Nutrient Supplementation on the Biodegradation of Naphthalene

In order to determine whether the availability of nitrogen and phosphorous might limit the uptake of naphthalene, the degradation of this hydrocarbon by free cells in seawater, with and without nutrient supplementation, was investigated (Figure 1, Table 2). The results at 30°C revealed that an initial naphthalene concentration of 25 mM was almost completely degraded (93%) within the first 6 days of incubation in samples supplemented with nitrogen and phosphorous, whereas in the nonsupplemented samples, only 42% of the naphthalene was consumed after that time. At temperatures of 16°C, and with an initial naphthalene concentration of 10 mM (conditions more similar to those found in marine environments), the presence of such nutrients was also seen to affect biodegradation, although to a lesser extent. So, in samples supplemented with nitrogen and phosphorous 55% the naphthalene was degraded, whereas in the non supplemented samples the degradation was only 34%.

According to these results, naphthalene was not toxic for *P. stutzeri* 19SMN4 when the only source of phosphorous and nitrogen came from seawater and supplementing seawater with phosphorous and nitrogen is important in order to obtain the highest possible degree of biodegradation. Accordingly, all ensuing biodegradation experiments were performed supplementing the seawater with sources of nitrogen and phosphorous.

TABLE 2 **Absolute Values Corresponding to Figures 1, 2, 3, and 4**

		C_0^a	Day 2	Day 3	Day 5	Day 6	Day 7
Free cells 16°C NP[b]	C in seawater (mM) Naphthalene degraded, mM and (%)	26.50 ±0.162	25.30 ± 0.639 1.2 (4.5%)		22.6 ± 0.250 3.9 (14.7%)	20.50 ± 0.357 6 (22.6%)	
Free cells 30°C	C in seawater (mM) Naphthalene degraded, mM and (%)	25.64 ± 0.213	19.96 ± 0.271 5.2 (20.1%)	19.10 ± 0.494 6.0 (23.5%)		14.44 ± 0.4008 10.7 (41.7%)	
Free cells 30°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	25.13 ± 0.251	17.79 ± 0.005 7.3 (29.2%)	12.15 ± 0.587 13.0 (51.6%)		1.68 ± 0.648 23.4 (93.3%)	
Free cells 16°C	C in seawater (mM) Naphthalene degraded, mM and (%)	9.62 ± 0.013	8.31 ± 0.637 1.31 (13.6%)	6.81 ± 0.294 2.8 (29.2%)		6.27 ± 0.021 3.3 (34.8%)	
Free cells 16°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	9.62 ± 0.013	6.87 ± 0.121 2.7 (28.6%)	5.03 ± 0.002 4.6 (47.7%)		4.30 ± 0.165 5.3 (55.3%)	
Immobilized cells (3600 cP) 16°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	26.62 ± 0.473		18.65 ± 0.361 8.0 (29.9%)	18.19 ± 0.133 8.4 (31.7%)	17.53 ± 0.034 9.1 (34.1%)	16,17 ± 0,524 10.4 (39.2%)
Immobilized cells (3600 cP) 16°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	9.42 ± 0.369		3.91 ± 0.305 5.5 (58.5%)			1.90 ± 0.229 7.5 (79.8%)
Immobilized cells (3600 cP) 30°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	25.94 ± 0.022	17.15 ± 0.069 8.8 (33.9%)	14.29 ± 0.069 11.6 (44.9%)		0.03 ± 0.042 25.9 (99.9%)	
Immobilized cells (240 cP) 16°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	25.72 ± 0.066		19.19 ± 0.064 6.5 (25.4%)	18.12 ± 0.191 7.6 (29.5%)		17.35 ± 0.088 8.37 (32.5%)
Immobilized cells (240 cP) 16°C NP	C in seawater (mM) Naphthalene degraded, mM and (%)	9.5 ± 0.107		3.63 ± 0.307 5.9 (22.8%)			1.598 ± 0,287 7.9 (83.0%)

[a]Initial concentration of naphthalene.
[b]Medium supplemented with nitrogen and phosphorous.

Effect of Temperature

The biodegradation of naphthalene was examined at 30°C and 16°C in order to test the effect of temperature on the process. Temperatures were respectively chosen according to the optimal growth conditions of *Pseudomonas* and the mean seawater temperature recorded for the Galician Coast. The results at incubation temperatures of 30°C show that both free and immobilized cells degraded almost all the hydrocarbon present in the culture medium with an initial concentration 25 mM within 6 days, whereas at 16°C only 22% was degraded in the case of free cells, and 34% in the case of immobilized cells (Figure 2, Table 2). The fact that at the same temperature immobilized cells showed higher degradation rates may be due to the diffusional limits provided by alginate, which protects the bacterium from substrate inhibition (Abou Seoud and Maachi, 2003). Biodegradation was much slower at 16°C than at 30°C. This could be due to both the solubility and, hence, the bioavailability, of naphthalene and to the rates of hydrocarbon metabolism, which decrease in parallel with decreasing temperatures (Bossert and Bartha, 1984; Shiu and Mackay, 2006).

After 3 days of incubation at 30°C, the medium turned brown; this could be due to the accumulation of cathecol, an intermediate product in the degradation of naphthalene. According to Park et al. (2004), a brown pigmentation appears as a result of the nonenzymatic auto-oxidation of cathecol and other related compounds.

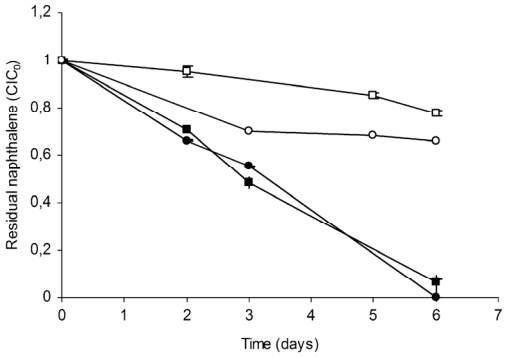

FIGURE 2 Biodegradation of a 25 mM initial concentration of naphthalene at 30°C (*filled symbols*) and 16°C (*open symbols*) by free (—■—,—□—) and immobilized cells in alginate with viscositie of 3600 cP (—●—,—○—) in seawater with nutrients. Error bars represent the *SD* of duplicate replicates. The *SD* is usually smaller than the symbol.

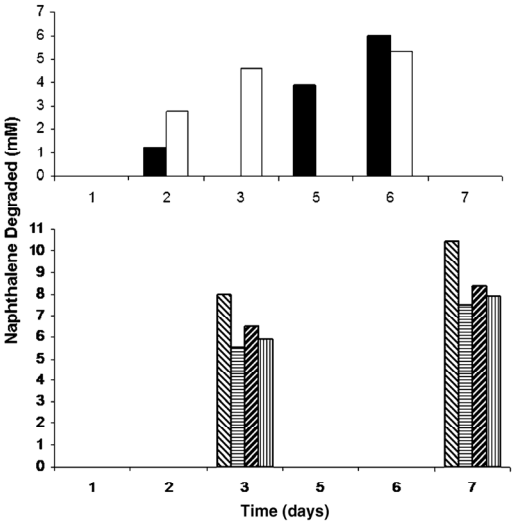

FIGURE 3 (*A*) Naphthalene degraded (mM) by free cells in seawater with nutrients at 16°C of a initial concentration of naphthalene: ■25 mM and □10 mM. (*B*) Naphthalene degraded (mM) in seawater at at 16°C with nutrients by immobilized cells in alginate with viscositie of 3600 cP of a initial concentration of naphthalene 25 mM and 10 mM and immobilized cells in alginate of 240 cP of a initial concentration of naphthalene 25 mM and 10 mM.

Effect of the Initial Concentration Of Naphthalene

When the initial concentration of naphthalene in the medium was reduced from 25 to 10 mM, the final degradation by free cells remained unaffected (Figure 3, Table 2). Thus, after 6 days of culture, 5.3 and 6 mM of the initial concentrations of 10 and 25 mM were respectively degraded. Also, in the case of the immobilized cells the concentration of naphthalene degraded with the two concentrations tested was similar: approximately 8 mM in the case of cells entrapped in alginate with a viscosity of 240 cP for both concentrations and 10.4 and 7.5 for the cells entrapped in alginate of 3600 cP with a concentration of 25 and 10 mM, respectively. These results show that possible substrate inhibition is not present when the initial concentration is increased from 10 to 25 mM. Lower degradation rates were observed after 3 days of culture. This could be due to the accumulation of metabolites in the medium, which slows down degradation. After 7 days of culture of immobilized cells with an initial concentration of naphthalene of 25 mM, the pH of the medium fell from 7.25 to 5.64, 7.0 being the optimal pH for the activity

of naphthalene dioxygenase (Dorn et al., 2003). This decrease in pH may be due to the NH_4NO_3 used as a nitrogen source to supplement the medium, because in a study by Aranha and Brown (1981) it was suggested that the use of this nitrogen source may lead to the accumulation of salicylic acid, which is an intermediate product of naphthalene catabolism, and hence to a reduction in the pH in the medium.

Effect of Alginate Viscosity

Alginate of three different viscosities was used to test the viability of entrapped cells and the degradation of naphthalene, as described in Materials and Methods. The shape of the beads was more spherical and regular in the case of those made with 3600 cP alginate. The 31.4 cP alginate afforded highly irregular beads, which were fragmented after 7 days of culture, whereas beads formed with 240 and 3600 cP alginate did not display any structural changes or damage after that time. These findings led us to discard the 31.4 cP alginate in further experiments. The initial concentration of naphthalene used for these experiments was 10 mM because this concentration was more similar to the real levels of naphthalene in contaminated seawater. Figure 4 shows that the viscosity of alginate does not affect the degradation rate of the cells. Thus, after 7 days of culture, beads made with both alginate types had degraded approximately the same amount of naphthalene present in the medium.

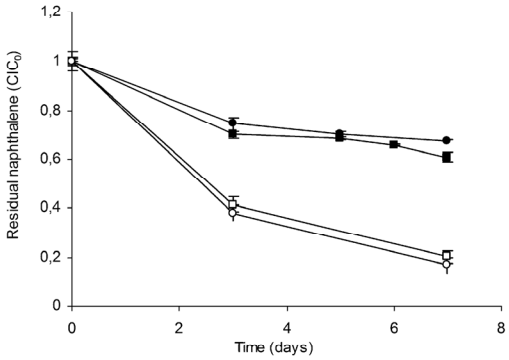

FIGURE 4 Biodegradation of a 25 mM (*filled symbols*) and 10 mM (*open symbols*) initial concentration of naphthalene by immobilized cells in alginate with viscosities of 3600 cP (—■—,—□—) and 240 cP (—●—,—○—) grown in seawater with nutrients. Error bars represent the *SD* of duplicate replicates. The SD is usually smaller than the symbol.

TABLE 3 Effect of Alginate Viscosity on the Viability of Immobilized Cells Growth in Seawater with Nutrients and 10 mM Naphthalene at 16°C

Inoculum		Viable cells Day 3	Viable cells Day 7
9.2×10^8 CFU/2 g	Immobilized cells (3600 cP)		
	Beads (CFU/2 g)	4.6×10^7	6.0×10^7
	Medium (CFU/ml)	ND[a]	8.0×10^2
9.9×10^8 CFU/2 g	Immobilized cells (240 cP)		
	Beads (CFU/2 g)	2.9×10^7	6.6×10^7
	Medium (CFU/ml)	3.2×10^4	8.2×10^4

[a]Not determined.

In order to check the cellular retention of the beads, the number of viable cells in the beads and in the medium was analysed after 3 and 7 days (Table 3). According to the results obtained, after 3 days the number of viable cells in the beads was reduced. This is probably due to an initial loss of cells caused by cellular death or exit to the culture medium. After 7 days culture, the number of viable cells in the beads was the same for both 240 cP and 3600 cP alginate. However, beads formed with high-viscosity alginate were more efficient at retaining cells, since the number of viable cells present in the medium was lower (8.0×10^2CFU/ml) than in cultures with lower-viscosity alginate beads (8.2×10^4 CFU/ml).

Through this work, we have found a practical difficulty associated with the naphthalene extraction: due to its low solubility in water and high surface adherence, representative sampling from the cultures was impossible. Hence, we decided entire cultures for the determination of naphthalene concentrations.

The final conclusions of this study showed that (i) the biodegradation ratio was highly affected by temperature and nutrient supplementation of the culture medium. The tested naphthalene concentrations (25 and 10 mM) either have no toxic effects, or not inhibit the biodegradation, although the seawater was not supplemented with additional nitrogen and phosphorous sources; (ii) The alginate viscosity used for cell immobilization has not important effects on the biodegradation rates, but it does affect the cell release to the culture medium.

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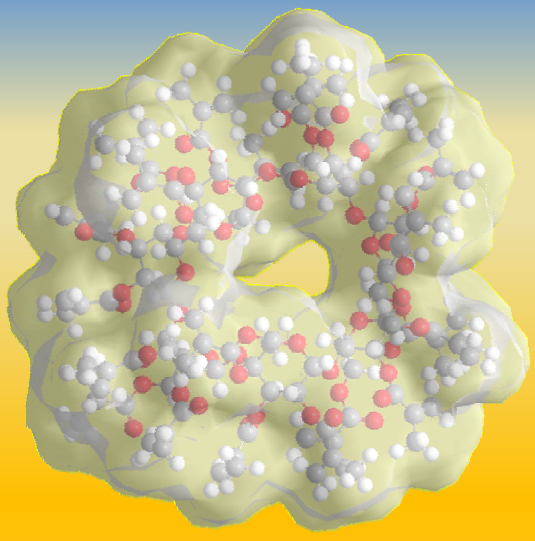
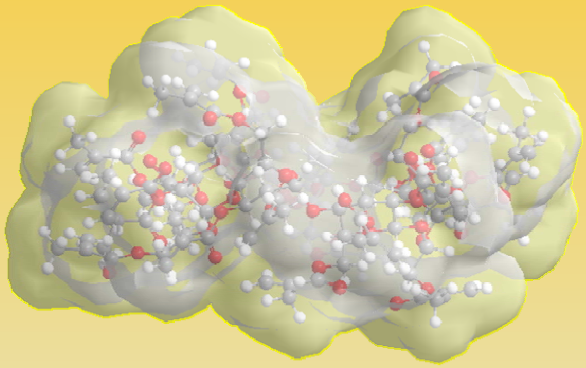


El diseño de sistemas de liberación de fármacos adaptados a las propiedades fisicoquímicas y farmacológicas de los fármacos y capaces de regular su liberación en respuesta a la evolución de ciertos procesos patológicos despierta un interés creciente. Las ciclodextrinas resultan particularmente atractivas para el desarrollo de dispositivos avanzados por su capacidad para formar complejos de inclusión con moléculas de naturaleza muy diversa. Además, la posibilidad de unión a una gran variedad de estructuras poliméricas abre interesantes perspectivas para el desarrollo de sistemas hidrofílicos capaces de incorporar fármacos, hidrofóbicos o hidrofílicos, y de cederlos de forma controlada. El presente trabajo se ha planteado con el objetivo de mejorar la capacidad de hidrogeles de polihidroxietilmetacrilato, de uso habitual en el campo de la biomedicina como componentes de lentes de contacto e implantes, para cargar fármacos y regular su cesión, incorporando ciclodextrinas al entramado polimérico. Se pretende combinar en un mismo entramado las propiedades de estos hidrogeles acrílicos, con la capacidad de las ciclodextrinas para formar complejos de inclusión. Para ello se abordaron dos aproximaciones diferentes para incorporar la ciclodextrina en el entramado polimérico: copolimerización o anclaje en el entramado preformado. Los materiales desarrollados se caracterizaron en cuanto a contenido en ciclodextrinas, propiedades superficiales, mecánicas, de hinchamiento y claridad óptica y citocompatibilidad. También se evaluó la capacidad de incorporación y de control de la cesión de fármacos antiinflamatorios y antimicrobianos. Los resultados obtenidos prueban que los materiales desarrollados pueden ser útiles como componentes de lentes de contacto y otros productos sanitarios medicados.



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